

EXTRACELLULAR DNA IN SOIL SYSTEMS:
STABILIZATION/DEGRADATION DYNAMICS AND IMPACTS ON
MICROBIAL COMMUNITY ANALYSIS

A Thesis

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ABSTRACT

Extracellular DNA (eDNA) is any single- or double-stranded DNA molecule not contained within a cell. This eDNA is ubiquitous in soils, where it arises from either cell lysis or DNA secretion, and where it may persist for many years. Interest in the issue of soil eDNA has recently been piqued in the fields of molecular biology and microbial ecology because eDNA has the potential to bias sequence-based estimates of microbial community composition, inflate measures of alpha and beta diversity, and interfere with the detection of community shifts over time.

In the first chapter, we examine the impacts of soil moisture, soil temperature, agricultural management, and habitat type on the degradation/persistence dynamics of eDNA in soil microcosms, using a synthetic eDNA marker which was traceable with both sequence-specific qPCR and 16S rRNA community sequencing. We found that despite very rapid degradation within the first week, a small fraction ($< 1\%$) of the eDNA standard remained detectable with qPCR throughout the experiments (39 - 77 days). This suggests that eDNA may be indefinitely stabilized within soil. We also found that degradation/stabilization dynamics differed across gradients of environmental conditions and soil characteristics, with initial degradation rate (within the first week) being positively correlated with soil moisture, temperature, and tillage intensity, but negatively correlated with soil organic matter content. Longer-term stabilization (≥ 39 days) of the eDNA standard was highest at low moisture, low temperature, and low tillage intensity, but was not significantly correlated with soil organic matter. Additionally, among agricultural, forest, and meadow soils we found

that forest soils had the slowest initial degradation rate, and meadow soils had the most stabilization of the eDNA standard. The eDNA standard was detectable by qPCR at all time-points for all treatments, but within the first week became only inconsistently detectable with high-throughput sequencing, the eDNA standard having dropped below the limit of detection for 16S rRNA gene sequencing. The time to disappearance below the sequencing limit of detection was calculated as ranging from 0.9 - 19.4 days, depending on treatment conditions. Therefore, we conclude that the ability of stabilized soil eDNA to bias estimates of microbial community structure depends on the sensitivity of the detection method and the objectives of the experiment.

In the second chapter, we place the first chapter into a wider context by reviewing the impacts of eDNA on estimates of microbial communities in soils, and discussing evidence that eDNA-driven bias is or is not a problem in community structure estimates. We discuss the factors that influence the degradation/stabilization dynamics of eDNA in soils, techniques to reduce eDNA-driven bias, potential opportunities to exploit eDNA as a powerful tool for microbial community characterization, and important directions for future eDNA-related research.

Taken together, this work constitutes a contribution of new knowledge and insight to the field of soil microbial ecology. This research and analysis will improve the ability of researchers to accurately characterize microbial communities using culture-independent methods, while understanding the extent to which eDNA may introduce bias to those estimates.

BIOGRAPHICAL SKETCH

Sara Hope Sirois grew up in rural Spafford, NY as the only child of the late Wayne and Connie Sirois. From an early age, Sara was fascinated by the natural world. She loved nothing more than exploring the hills above Lake Skaneateles with her two trusty, but not particularly well-groomed, Samoyeds. Supported by her friends and family (and the family pets, of course), Sara graduated *magna cum laude* in 2011 from Smith College in Northampton, MA, where she received a BA with honors in Biology, and a minor in Chemistry. At Smith, she was advised by Dr. Laura A Katz, Dr. Robert B. Merritt, and Dr. Robert Newton. With their guidance, she completed independent research characterizing bacterial communities, with special emphasis on sulfate-reducing bacteria, in the sediments of beaver ponds. After college, Sara spent the summer researching ovarian cancer treatments in the lab of Dr. Elise Kohn at the NIH in Bethesda, MD. She then volunteered with the Peace Corps in Sierra Leone as a math and science teacher, and upon her return to the United States, pursued her interest in organic agriculture. After completing agricultural apprenticeships at both Heifer Farm in Rutland, MA and Caretaker Farm in Williamstown, MA, she returned to academia as a graduate student. She joined the lab of Dr. Daniel H. Buckley at Cornell University in the fall of 2016. After leaving Cornell, Sara plans to establish her own organic farm, where she can continue to roam the hills and marvel at the unseen wonders in the soil at her feet.

Dedicated to my parents, Connie and Wayne Sirois.

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CHAPTER 1

EXTRACELLULAR DNA DEGRADATION/STABILIZATION DYNAMICS IN SOIL VARY ACROSS GRADIENTS OF MOISTURE, TEMPERATURE, HABITAT TYPE, AND MANAGEMENT REGIME

1.1 *Abstract*

Extracellular DNA (eDNA), defined as any DNA not contained within a cell, is ubiquitous in soils and may persist for many years. When using high-throughput gene sequencing and other culture-independent methods eDNA may bias microbial community analyses, potentially inflating measures of alpha and beta diversity and challenging our ability to interpret temporal trends. To assess the impacts of eDNA dynamics on estimates of community composition, we examined the impacts of soil moisture, soil temperature, agricultural management, and habitat type on the degradation/persistence dynamics of eDNA in silty loam to silty clay loam soils. Synthetic eDNA was added to soil microcosms, and its disappearance over time was measured using both high-throughput sequencing and qPCR. We found that eDNA was degraded rapidly, being reduced to $6.4 \times 10^{-3} \pm 7.6 \times 10^{-3}$ of initial gene copies by day 7 (a 99.4 ± 0.8 % reduction). Despite its rapid degradation, a small fraction ($< 1\%$) of the eDNA remained detectable throughout the experiments (39 - 77 days) suggesting indefinite stabilization within soil. Rates of degradation and levels of stabilization depended on environmental conditions and soil characteristics. The

eDNA degradation rate was positively correlated with moisture and temperature, but negatively correlated with soil organic carbon content. The ultimate stabilization of eDNA was highest at low moisture and temperature, but stabilization (≥ 39 days) exhibited no relationship with soil organic carbon. We also observed that both tillage regime and habitat type impacted eDNA dynamics. Tilled soils had higher rates of degradation and less stabilization than no-till soils. In addition, among agricultural, forest, and meadow habitats we observed that forest soils had the slowest degradation rate, and meadow soils had the greatest stabilization of eDNA. While eDNA was detectable by qPCR in all treatments across all time-points, it began to drop below the limit of detection for 16S rRNA gene sequencing in less than one week, with the time to disappearance in sequencing libraries being calculated as ranging from 0.9 - 19.4 days. We conclude that small amounts of eDNA may persist in soils indefinitely, and that the degradation rate and the amount of stabilization will vary with moisture, temperature, and habitat characteristics, but that the ability of this persistent eDNA to impact microbial community analyses will depend on method sensitivity and experimental objectives.

1.2 Introduction

Extracellular DNA (eDNA) is any DNA not contained within a cell. Cells release DNA into the environment through secretion or upon cell death and lysis (reviewed in Pietramellara et al. 2009). This eDNA is ubiquitous in soils, and can reach levels of 2ug/g (Niemeyer and Gessler 2002) and compose a major portion of the sequenceable soil DNA pool (Carini et al. 2017; Lennon et al. 2017). Because

relatively intact eDNA may persist for months or years (Gebhard and Smalla 2006), eDNA has the potential to obfuscate microbial community estimates obtained through culture-independent methods. Because high-throughput sequencing as a method to estimate microbial communities has expanded greatly in recent years, the potential impacts of eDNA on apparent community structure have come under increasing scrutiny.

It is widely accepted that eDNA is present in most environmental systems, but there is disagreement about how much bias eDNA introduces to soil community data. Carini, et al. (2017) concluded that eDNA profoundly impacted apparent microbial community structure and richness. In contrast, Lennon et al. (2017) found that even when eDNA was present in large quantities, it did not significantly affect bacterial community structure, richness, evenness, or diversity. Based on simulated data, they also demonstrated that the proportion of the total DNA pool composed of eDNA did not control the degree to which bias was introduced to community estimates (Lennon et al. 2017). Rather, differences between degradation rates of eDNA and intracellular DNA (iDNA) pools controlled how much bias was introduced (Lennon et al. 2017). More bias arose when there was either differential degradation of eDNA between species, or differential degradation of the eDNA that comprised a larger portion of the total eDNA pool (Lennon et al. 2017). These observations indicate that the degradation/stabilization dynamics of eDNA in soils may regulate the extent to which estimates of microbial community composition are potentially biased by eDNA. For this reason, it is important to understand the degradation/persistence dynamics of eDNA in soils, and to elucidate the controls on eDNA dynamics.

Once eDNA enters the soil, its fate is controlled by both intrinsic and extrinsic properties, which may influence the degradation/persistence dynamics of soil eDNA. The intrinsic controls on eDNA dynamics include DNA source (Gulden et al. 2005; Pietramellara et al. 2009), G+C content (Hofreiter et al. 2001), molecular purity (Nielsen et al. 2000), and molecular weight (Ogram et al. 1988; Ogram et al. 1994; Pietramellara et al. 2009). Extrinsic conditions influencing eDNA degradation/persistence include soil mineralogy (Greaves and Wilson 1969; Lorenz and Wackernagel 1987; Ogram et al. 1988; Levy-Booth et al. 2007; Pietramellara et al. 2009; Gardner and Gunsch 2017), organic components (Ogram et al. 1988; Crecchio and Stotzky 1998; Saeki et al. 2011), pH (Levy-Booth et al. 2007), electrostatics (Carini et al. 2017), temperature (Widmer et al. 1996; Gulden et al. 2005), and moisture (Widmer et al. 1996).

Although our understanding of factors affecting the fate and dynamics of eDNA is growing, few studies provide a quantitative understanding of the stabilization/degradation dynamics of eDNA in natural soil systems through controlled experiments. In order to better understand the dynamics of eDNA degradation and stabilization in soils and the impacts of eDNA on apparent bacterial community in soils, the present study used a synthetic ds-eDNA fragment added to soil microcosm incubations. We compared eDNA degradation in soils from three habitats (agriculture, meadow, and forest) and from maize agricultural systems under distinct management schemes (factorial combinations of till and no-till, and biomass removed and returned), as well as in soils maintained under distinct environmental conditions (levels of moisture and temperature). The degradation of the added eDNA over the

course of 5-10 weeks was quantified using both gene-specific qPCR and high-throughput 16S rRNA community sequencing. These detection methods were compared.

As described in previous studies (Widmer et al. 1996; Morrissey et al. 2015), we predicted that eDNA would be degraded rapidly after addition to soil, but that degradation rates would decline over time. We predicted that eDNA degradation would be largely biotic in nature, and that stabilization is likely a result of physical protection in the form of occlusion, adsorption, or diffusion limitation. Because soil conditions (mineralogy, organic matter, pH, etc.) are known to influence the fate of eDNA, we predicted that there would be differential eDNA degradation/stabilization dynamics across gradients of moisture, temperature, habitat, and soil management regime. The results of this study help to clarify the conditions under which eDNA might bias bacterial community estimates, and under which conditions the presence of eDNA can be assumed to interfere minimally with community estimates.

1.3 *Methods*

Unless otherwise noted, all values throughout the text are expressed as the average \pm standard deviation.

1.3.1 *Development of eDNA Standard:*

For the purposes of these experiments, a single eDNA standard was needed for both qPCR and for 16S rRNA gene sequencing, so that the standard could be detected in DNA extracted from a single sample, analyzed using both techniques. For 16S

sequencing, a fragment of the *Streptococcus mutans* genome, coding for the *htrA* gene, was altered to include 515f/806r primer sites (F:GTGCCAGCAGCCGCGGTAA, R:ATTAGATACCCGGGTAGTCC) (Caporaso et al. 2012). Because the *S. mutans* *htrA* gene is also amplifiable with species-specific primers (F:TCGCGAAAAAGATAAACAACA, R:CTAACCAACTGTGAAGGGGC) the fragment was detectable by both community sequencing and by species-specific qPCR primers (Chen et al. 2007). Care was taken to locate the primer sites such that amplicons produced from the 16S primer sites would be equivalent to their native counterparts in length and GC content, as well as unlikely to induce self-annealing. The designed eDNA standard sequence was ordered as a synthetic oligonucleotide fragment, re-suspended in molecular-grade water to 10ng/uL, and applied to soils without further alteration (GBlocks gene fragments, IDT). To verify that no *S. mutans* was present natively in our soils, which would interfere with qPCR data, each soil DNA was subjected to PCR with the species-specific *htrA* primers. No amplification occurred, indicating that our soils are natively free of *S. mutans* (unpublished data).

1.3.2 Sampling Sites:

The soils used for experiments to determine the effect on eDNA degradation of moisture, temperature, and management were all obtained from a long-term tillage trial conducted at the Miner Institute for Agricultural Research in Chazy, NY (Clinton County, 44°53.13'N, 73°28.40'W). These plot are part of a long-term maize management trial, established in 1973, which compares fully factorial combinations of tillage and biomass replacement/removal, all within the same Raynham silt loam soil

type. The tillage treatments are conventional tillage (moldboard plowed and disked) vs no-till, and the biomass treatments are the harvesting of all above-ground biomass (biomass harvested) vs the harvesting only of the maize grain with return of the shoots to the soil (biomass returned). Thus, there are four treatments: tilled with biomass harvested (PTH), tilled with biomass returned (PTR), no-till with biomass harvested (NTH), and no-till with biomass returned (NTR). All treatment combinations are applied to four replicate plots (6 x 15.2 m), which are arranged in a randomized block. See previous studies from the same site for greater detail (Hsu and Buckley 2009; Koechli 2016).

Soils were collected from all plots as approximately 20 cores (2.5 cm diameter, 5 cm depth) at random locations throughout the plots, including both rows and furrows. Samples were taken on May 28, 2017, very shortly after planting (and plowing, when applicable), but before emergence of the maize seedlings. Cores from each individual plot were transported on ice, homogenized, sieved (2 mm), and air-dried at room temperature. Samples were stored at room temperature after drying.

The soils used for experiments to determine the effect on eDNA degradation of habitat type were collected from the Monkey Run Natural Area in Ithaca, NY (42°28'13.8"N 76°25'50.8"W) on November 8, 2017. The plots represent a successional series ranging from in-use agricultural plots through secondary growth forest on silt loam or silty clay loam soils. Samples were taken along 3 transects, each cutting across either agricultural field (cover crop), successional meadow, or secondary forest. Within each habitat type, four replicate locations were chosen at intervals, and approximately 3 cores were collected at each location, resulting in four

replicates from each habitat type. Cores from each location within a habitat were transported on ice, homogenized, sieved (2 mm), and air-dried at room temperature. Samples were stored at room temperature after drying.

1.3.3 Determination of Soil Water-Holding Capacity:

The water-holding capacity (WHC) of each air-dried soil was determined per the Keen-Raczkowski box method (Keen and Raczkowski 1921). Perforated aluminum weigh boats were used in lieu of copper boxes, and rather than allow soils to absorb water overnight, boats were removed from water after soil water uptake had ceased (~10 minutes). Water-holding capacity was determined gravimetrically. For these experiments, the water-holding capacity of soil from each replicate plot was determined, and the capacities were averaged across management or habitat type.

1.3.4 Determination of Soil Carbon, Nitrogen, and pH:

Soil pH was measured using the 1:2 soil:water method and a calibrated pH probe, and total soil carbon and nitrogen were determined by the Cornell Nutrient Analysis Lab on air-dried and ground samples using a LECO Treu Mac CN-2000 analyzer (LECO Instruments, Lansing, MI) as previously described (Berthrong et al. 2013). See Table 1 for soil chemistry summaries.

Table 1: Summary of soil chemical properties. Subscript letters in each row indicate significant differences (Tukey's HSD, $p < 0.05$) between treatment groups. C and N data for Chazy soils have been reproduced in part from (Koechli 2016).

Treatment/Habitat	pH	%Carbon	%Nitrogen	C:N
agriculture	4.95 ± 0.36^{ab}	3.16 ± 0.46^b	0.25 ± 0.04^b	12.89 ± 0.64^a
forest	4.36 ± 0.22^a	6.00 ± 0.73^d	0.35 ± 0.02^c	17.22 ± 2.73^b
meadow	5.35 ± 0.26^b	4.46 ± 0.44^c	0.36 ± 0.02^c	12.37 ± 0.70^a
No-till + biomass harvested (NTH)	6.89 ± 0.44^c	2.13 ± 0.39^{ab}	0.18 ± 0.04^b	11.58 ± 0.32^a
No-till + biomass returned (NTR)	6.89 ± 0.27^c	2.77 ± 0.48^b	0.23 ± 0.03^b	12.20 ± 0.68^a
Tillage + biomass harvested (PTH)	7.53 ± 0.17^d	1.50 ± 0.26^a	0.10 ± 0.02^a	14.36 ± 1.12^{ab}
Tillage + biomass returned (PTR)	7.59 ± 0.08^d	1.62 ± 0.40^a	0.11 ± 0.01^a	14.36 ± 2.02^{ab}

1.3.5 Microcosm Experimental Design:

To determine the effect of a given treatment on eDNA degradation, soil was weighed (0.50 g) into microcosms. Each microcosm received 10 ng synthetic eDNA standard in enough water to achieve a given water-holding capacity (WHC) of the soil (see specifics for each experiment below). Microcosms were left uncapped in a large insulated chamber to prevent CO₂ build-up within the microcosm upon re-wetting of soil. Chambers were held at constant temperature, and were weighed every 1-3 days, and any moisture lost was replaced. Samples were collected at 0 hours (immediately after application of eDNA standard) and at various subsequent time-points. Upon collection, samples were frozen at -20°C until DNA extraction and downstream analysis.

Effect of soil moisture:

The soils used were those from the Chazy NTH treatment (4 replicate plots). Microcosms were maintained at room temperature. For 20% WHC samples WHC was maintained at 17.4 ± 3.1 %, for 50% WHC samples WHC was maintained at 46.9 ± 3.5 %, and for 80% WHC samples WHC was maintained at 78.1 ± 3.2 %. Samples were collected at 0, 1, 2, 3, 4, 6, 8, and 11 weeks.

Effect of soil temperature:

The soils used were those from the Chazy NTH treatment (4 replicate plots). Chambers were placed either at 4°C, 20°C, or 36°C. For 4°C samples WHC was maintained at 48.7 ± 1.8 %, for 20°C samples WHC was maintained at 45.8 ± 5.7 %, and for 36°C samples WHC was maintained at 45.4 ± 6.5 %. Samples were collected at 0, 1, 2, 3, 4, and 6 weeks.

Effect of soil management:

The soils used were those from the Chazy management treatments (PTH, PTR, NTH, NTR), 4 replicate plots within each of the 4 treatments. Microcosms were maintained at room temperature. Samples of the PTH treatment were held at 48.3 ± 1.7 %WH. Samples of the NTH treatment were held at 48.5 ± 1.6 % WH. Samples of the PTR treatment were held at 48.8 ± 1.5 % WH. Samples of the NTR treatment were held at 48.4 ± 1.5 % WH. Samples were collected at 0, 1, 2, 3, 4, and 5.5 weeks.

Effect of habitat type:

The soils used were those from Monkey Run (4 replicates from each habitat type). Microcosms were maintained at room temperature. Agriculture soil samples were held at 48.8 ± 1.8 % WH, forest soil samples were held at 48.5 ± 2 % WH, and meadow soil samples were held at 48.9 ± 1.4 % WH. Time-point samples were collected at 0, 1, 2, 3, 4, 6, and 8 weeks.

Determination of biotic vs. abiotic protection/degradation of eDNA:

The soils used were those from the Chazy NTR and PTH treatments (4 replicate plots within each treatment). Dry soils (0.50 g) were weighed into tubes and autoclaved three times in 90 minute cycles, being allowed to cool completely between each autoclaving cycle. Matching replicates were left un-autoclaved and at room temperature. To each tube (both autoclaved and un-autoclaved) was applied 3.4 ng eDNA standard. Tubes of soil were maintained at room temperature and 50% WH. Samples were collected at 0, 1, 2, and 3 weeks.

1.3.6 Detection of eDNA Standard by qPCR and Sequencing:

From each microcosm sample, total soil DNA was extracted using the PowerSoil kit (Mo Bio/Qiagen). DNA concentration was quantified using the PicoGreen quantification system (Invitrogen, Carlsbad, CA, USA).

To quantify the copy numbers of the eDNA standard present in the extracted DNA samples, qPCR was performed using primers specific to *S. mutans* (Chen et al. 2007). Each 25 μ L reaction contained 1x High-fidelity HotStart PCR Master Mix

(New England Biolabs), 1 μ L each primer (100 uM, Integrated DNA Technologies), and 0.625 μ L 200x Picogreen reagent (Invitrogen), as well as 1 μ L template DNA (diluted 1:10 in water). Thermal cycling (Bio Rad CFX Connect) consisted of the following: denaturation at 95°C for 2 minutes, 40 cycles of amplification (95°C for 30s, 63°C for 30s, 72°C for 60s), a melt curve from 55°C-95°C for quality assurance of amplicons, and final elongation at 72°C for 5 minutes. Standard curves were produced by replacing the template DNA in the reaction with 1 μ L of serially diluted eDNA standard, with standard concentrations from 1×10^8 through 1×10^2 . To determine eDNA standard copy numbers in samples, the cycle threshold (Cq) at which each sample reached sufficient amplification to produce 50 RFU (baseline fluorescence) was compared against the standard curve made by plotting Cq of each standard against its known concentration. Each starting quantity of eDNA standard was corrected by the amount of dry soil from which the DNA was extracted (g), to yield units of copies per g dry soil (copies/g). All qPCR data were then expressed as “Fraction Remaining of eDNA Standard,” given by dividing the individual replicate copies/g by the mean copies/g of all replicates of the treatment to which the individual replicate pertains. All qPCR non-detects were handled by replacing the cycle threshold (Ct) value with that of 40, the maximum cycle, as previously described (Goni et al. 2009; Mar et al. 2009).

In addition to performing qPCR with primers specific to the eDNA standard, community sequencing was also performed on certain samples to determine whether the eDNA standard was detectable by sequencing analyses. The 16S rRNA genes was amplified with PCR using universal bacterial V4 region 515f/806r primers, barcoded

with dual-indexing tags as previously described (Kozich et al. 2013). The synthetic eDNA standard was designed to include these primer sites and to result in an amplicon of length equivalent to that of non-synthetic amplicons, and is therefore captured in 16S community data. Sequencing amplification reactions contained components in the same ratio as described above for qPCR, though thermal cycling consisted of the following: denaturation at 95°C for 2 minutes, 30 cycles of amplification (95°C for 30s, 55°C for 15s, 72°C for 10s), a melt curve from 55°C-95°C for quality assurance of amplicons, and final elongation at 72°C for 5 minutes. Each sample was amplified in triplicate, triplicate reactions were pooled, and PCR products were purified and normalized using the SequalPrep kit (Invitrogen), per the manufacturer's protocol. Normalized samples were combined, concentrated via vacuum centrifugation down to 200uL, and size-selected using agarose gel excision. Purified product was obtained using the Wizard SV Gel and PCR Clean-Up System (Promega), concentration was adjusted to 5 ng/uL, and product was submitted to the Cornell (Ithaca, NY) Core Facility for sequencing on the Illumina MiSeq platform, using V2 chemistry with 2 x 250 bp reads.

1.3.7 Determination of Limit of Detection and Limit of Quantification of qPCR:

Limit of detection (LoD) and limit of quantification (LoQ) were determined as previously outlined . In short, 24 replicate qPCR standard curves with concentrations of synthetic eDNA standard ranging from 0 copies/reaction to 1000 copies/reaction were prepared and cycled as described above. The LoD is the lowest DNA concentration at which >95% of reactions are positive, and the LoQ is the lowest DNA

concentration at which all reactions are positive and the coefficient of variance (CV) is below 35% (Forootan et al. 2017). The equation to determine CV is $CV(\%) = 100 * (\text{sample standard deviation} / \text{sample mean})$. For this qPCR system, the LoD and LoQ were both determined to be 1000 copies of synthetic eDNA standard per 25 μL PCR reaction.

1.3.8 Determination of Limit of Detection of High-Throughput Sequencing:

Limit of detection (LoD) for sequence data was determined *in silico*, using a Monte Carlo simulation ($n=1000$) selecting 2×10^4 sequences (representative of our sequencing depth) from a pool of 1×10^9 (representative of reasonable cell counts from 0.25 gram of soil) sequences with the percent sequence of interest ranging from 0.005% to 5%. As the lowest percent sequence of interest that was still found to be detectable at least 95% of the time, the LoD was determined to be 1.5×10^{-4} .

1.3.9 Sequence Analysis:

Amplicon sequence libraries were quality filtered and processed using QIIME2 (v.11) according to a general workflow (https://github.com/Roli-Wilhelm/Buckley_Lab_SIP_project_protocols/blob/master/sequence_analysis_walkthrough/QIIME2_Processing_Pipeline.ipynb; accessed January 2018). DADA2 was used to error-correct and cluster 16S rRNA gene libraries into operational taxonomic units (OTUs) at 1%. Classification of taxonomy was carried out with the QIIME2 feature classifier, trained with the 16S rRNA Greengenes database. Data were computationally rarefied by experiment (moisture experiment = 23,252 reads/sample,

temperature experiment = 23,210 reads/sample, management experiment = 14,874 reads/sample, habitat experiment = 22795 reads/sample) using phyloseq features in R (McMurdie and Holmes 2013). Synthetic eDNA standard sequence counts are presented as relative abundance. Raw sequencing data will be made available upon request. All metadata used in analyses can be found embedded in phyloseq objects (McMurdie and Holmes 2013), also available upon request.

1.3.10 Statistical Analysis:

Both standard ANOVA and a model fitting approach were used to analyze abundance of the eDNA standard in both qPCR and sequencing data. One-way ANOVA was used to evaluate comparisons between treatments within a single time-point, and Tukey's "Honest Significant Difference" test (Tukey HSD) was used to perform post-hoc analyses. Pearson's product-moment tests were used for evaluation of correlations. For sequence data this was sufficient, but for qPCR data, more sophisticated modeling analyses were performed. Specifically, for qPCR data evaluation of experimental results across time was performed using an exponential decay model fit to log-transformed data using a nonlinear least-squares regression. The model had three parameters: an initial value parameter (R_0), a decay parameter (LRC), and an asymptote parameter (ASYM). Time (t) is also included in the model, but is not a parameter subject to manipulation. R_0 indicates the eDNA fraction present at the initiation of the experiment (0 days), and was held constant across all treatments within an experiment. LRC is the natural logarithm of the decay rate. ASYM is equal

to the fraction of eDNA stabilized by the end of the experiment. The general equation of the model is shown below (Equation 1):

Equation 1:

$$ASYM + (R0 - ASYM) * \exp(-t * e^{LRC})$$

LRC and ASYM were either allowed to vary between treatment groups within an experiment, or were held constant between treatment groups. Parameters were estimated from models which provided the best fit to the data. Model parameter assumptions are shown in table 2 below. Model fit was evaluated using both the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) (Schwarz 1978). For both criteria, lower scores indicate better model fit. Models which failed to converge were rejected. Models were fit and confidence intervals for model parameters were obtained using the nlstools package in R (Baty et al. 2015). Confidence intervals were corrected to 95% by using the Bonferroni correction. All statistical analyses were performed in R, and code can be made available upon request.

Table 2: Model parameter assumptions for three parameters. The initial value parameter (R0), the decay parameter (LRC), and the asymptote parameter (ASYM), as well as the datasets which were best fit by the model as estimated with AIC and BIC.

model	R0	LRC	ASYM	By AIC, best fit for:	By BIC, best fit for:
Model A	Same	Different	Different	temperature, tillage, habitat, moisture*	temperature, tillage, habitat
Model B	Same	Same	Different	management	moisture*, management
Null model	Same	Same	Same	none	none

*AIC and BIC were not in agreement about which version of the model fit best for the moisture data. The model fits are not significantly different (p=0.11).

1.3.11 Time to Disappearance of eDNA standard from Sequencing Library:

The presence of eDNA will cause current estimates of microbial community composition to be influenced by previous states of the system, with the magnitude of antecedent effects driven by the quantity of eDNA inputs, the rate of eDNA turnover, and the potential for stabilization. We estimated the potential for eDNA to cause temporal delay in microbial community measurements as the time it takes for the eDNA standard to disappear from DNA sequencing libraries. This temporal delay was estimated by comparing modeled degradation rates determined by qPCR with the LoD for sequencing. This approach allowed us to determine the time required for eDNA to drop below the high-throughput sequencing LoD.

We first converted the sequencing LoD into the units of “fraction of eDNA standard remaining” (the y-axis units of the qPCR data models) by multiplying the sequencing LoD (in terms of relative abundance) by the qPCR copies at day 0 (in terms of fraction remaining), then dividing that number by the sequence relative abundance at day 0 (as determined with high-throughput sequencing). Using the modeled curves from the qPCR data, we then calculated the time at which the modeled curve for each treatment crossed the sequence LoD (expressed as fraction eDNA standard remaining).

1.4 RESULTS:

1.4.1 Experimental Parameters:

Assay parameters were standardized across microcosms (with the exception of independent variables) and the degradation of the eDNA standard was assayed by both

qPCR and high throughput DNA sequencing. A total of 20 ng eDNA standard g⁻¹ dry soil was added to each microcosm and this was found by qPCR to represent $2.1 \times 10^{10} \pm 2.7 \times 10^{10}$ gene copies per g soil, and $1.2 \times 10^{-2} \pm 8.9 \times 10^{-3}$ relative abundance of DNA sequences. The total amount of eDNA standard was held constant and measurements of eDNA standard at time zero did not vary between treatments within each experiment (unpublished data). The limit of detection for our qPCR assay was experimentally determined to be 1×10^3 gene copies per 25 μ L PCR reaction, (coefficient of variance of 3.47%). The limit of detection for sequence data was equal to a relative abundance of 1.5×10^{-4} of the total community.

1.4.2 Determination of biotic vs. abiotic protection/degradation of eDNA:

Most synthetic eDNA standard (> 99%) was degraded within the first week in un-autoclaved soils, but in autoclaved soils the eDNA standard was degraded much less readily, suggesting that most eDNA degradation in these soils is biotically-mediated (Figure 1). At time 0, the fraction eDNA standard remaining did not differ across treatments (1.00 ± 0.37 , one-way ANOVA, $p = 0.55$), but at all subsequent times the autoclaved soils had significantly different and greater eDNA stabilization than the un-autoclaved soils (one-way ANOVAs, all $F_{1,46} > 170$, all $p < 2 \times 10^{-16}$).

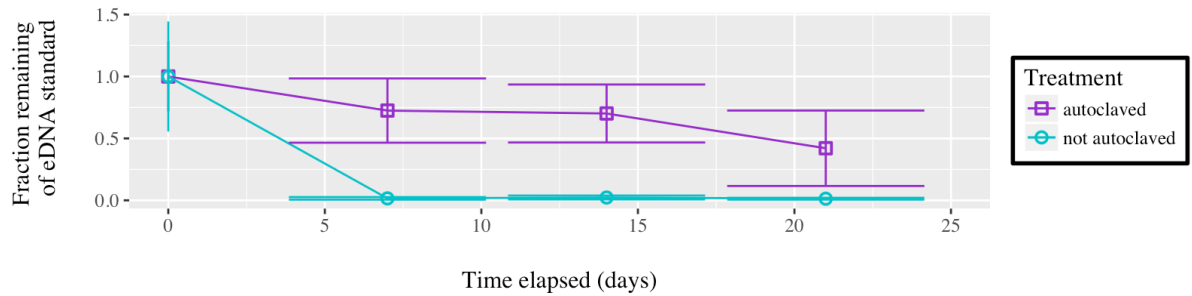


Figure 1: Synthetic eDNA degradation over time in autoclaved (purple squares) and un-autoclaved (blue circles) soils. Error lines represent standard deviation. There were significant differences in the amount of eDNA standard remaining at all times after day 0 (one-way ANOVAs, all $F_{1,46} > 170$, all $p < 2 \times 10^{-16}$).

1.4.3 Model fits of qPCR data:

AIC and BIC scores for each of the model fits are shown in Table 3 below.

Table 3: Model fits for each microcosm experiment qPCR dataset (moisture, temperature, management, habitat), along with AIC, BIC, and model ultimately chosen.

Experiment	AIC scores	BIC scores	Model chosen
Moisture	Model A: 189.70 Model B: 190.25 Null: 437.55	Model A: 219.00 Model B: 212.23 Null: 452.20	Model B*
Temperature	Model A: -81.16 Model B: -60.46 Null: 175.67	Model A: -54.16 Model B: -40.21 Null: 189.17	Model A
Management	Model A: non-convergence Model B: 162.67 Null: non-convergence	Model A: non-convergence Model B: 188.29 Null: non-convergence	Model B
Tillage	Model A: 154.71 Model B: 159.51 Null: non-convergence	Model A: 176.66 Model B: 177.81 Null: non-convergence	Model A
Habitat	Model A: 130.42 Model B: 140.52 Null: 184.63	Model A: 158.65 Model B: 161.69 Null: 198.75	Model A

*Given that AIC and BIC were not in agreement about which version of the model fit best for the moisture data, Model B was chosen because BIC showed a stronger magnitude of preference (6.77 points) versus AIC (0.55 points). The model fits are not significantly different ($p=0.11$).

1.4.4 Effects of moisture on eDNA dynamics:

Most synthetic eDNA standard (> 99%), as measured with qPCR, was degraded within the first week regardless of moisture, but detectable eDNA standard remained stabilized in all treatments across the entire 11 week incubation period (Figure 2). The qPCR Cq values for the samples were significantly different from those of the negative controls, both before imputing non-detects to Cq = 40 (Mann-Whitney U test, $W = 0$, $p = 4.0 \times 10^{-7}$), and after imputation (Mann-Whitney U test, $W = 36$, $p = 1.1 \times 10^{-5}$). At time 0, the fraction eDNA standard remaining did not differ across all 3 moisture treatments (1.00 ± 0.33 , one-way ANOVA, $p = 0.193$). Soil moisture content had a significant impact on the fraction eDNA standard remaining at 7 days (one-way ANOVA: $F_{2,33} = 44.5$, $p = 4.27 \times 10^{-10}$), with the 20% WHC treatment having the greatest fraction of eDNA remaining ($6.2 \times 10^{-3} \pm 2.9 \times 10^{-3}$), followed by the 50% WHC treatment ($1.2 \times 10^{-3} \pm 7.7 \times 10^{-4}$), and the 80% WHC treatment ($7.9 \times 10^{-4} \pm 4.4 \times 10^{-4}$).

Soil moisture content also had an impact on ultimate eDNA stabilization in soil (at final time-point), as estimated by model asymptotes. Stabilization of the eDNA standard was higher at 20% WHC (ASYM = $1 \times 10^{-2.5} \pm 1 \times 10^{-0.1}$, 95% CI) than at either 50% WHC or 80% WHC (95% CI: ASYM = $1 \times 10^{-3.4} \pm 1 \times 10^{-0.1}$ and $1 \times 10^{-3.5} \pm 1 \times 10^{-0.1}$, respectively), and this difference was significant (95% confidence intervals of the model did not overlap). The model was insensitive to differences in decay rate between moisture treatments (95% CI: LRC = -1.16 ± 0.21). However, based on measurements of initial rate of decay (the disappearance of eDNA standard over the first 7 days after addition to microcosm, rather than the modeled rate of

decay), the 20% WHC treatment had a significantly different rate of initial decay as compared to the 50% and 80% WHC treatments (one-way ANOVA, $F_{2,33} = 44.5$, $p = 4.3 \times 10^{-10}$, Tukey HSD, for 20% vs 50% and 80%, both $p < 1.0 \times 10^{-7}$).

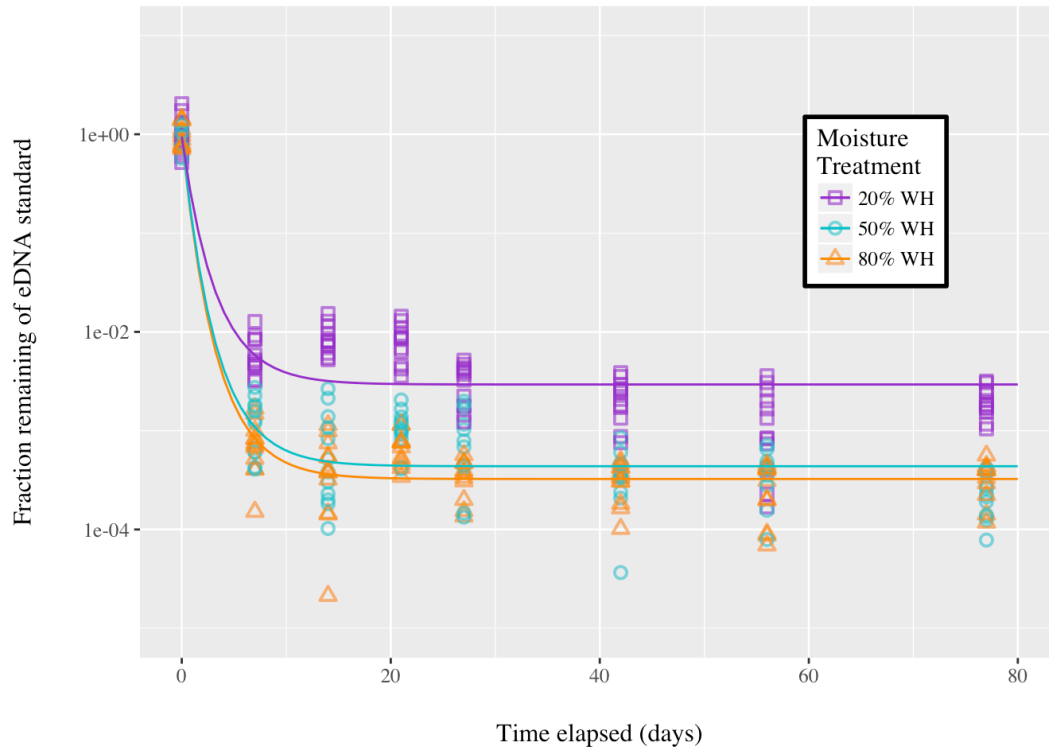


Figure 2: The qPCR data for synthetic eDNA degradation over time in 20% WHC (purple squares), 50% WHC (blue circles), and 80% WHC (orange triangles) treatments, with solid lines showing model predictions. There were no significant differences in the LRC (decay rate) term of the models, but there was more eDNA stabilization in the 20% WHC treatment as compared to the 50% and 80% WHC, a difference which was significant (95% CI did not overlap).

While the results from DNA sequencing of moisture experiment samples were generally consistent with those from qPCR, it was clear that sequencing had lower sensitivity than qPCR. The eDNA standard was observed to degrade rapidly in all treatments and fell below the limit of detection (the minimum concentration detectable >95% of the time) for DNA sequencing within 7 days of incubation. The standard

remained undetectable in the 50% and 80% WHC treatments throughout the experiment, though trace levels of eDNA standard ($< 8 \times 10^{-5}$ relative abundance) were detected at 3 of 7 time points in the 20% WHC treatment (Figure 3). This result suggests greater stabilization of eDNA in the 20% WHC treatment, consistent with qPCR results, but we did not observe a significant difference in stabilization between treatments as measured by DNA sequencing (multiple one-way ANOVA, all $p > 0.05$).

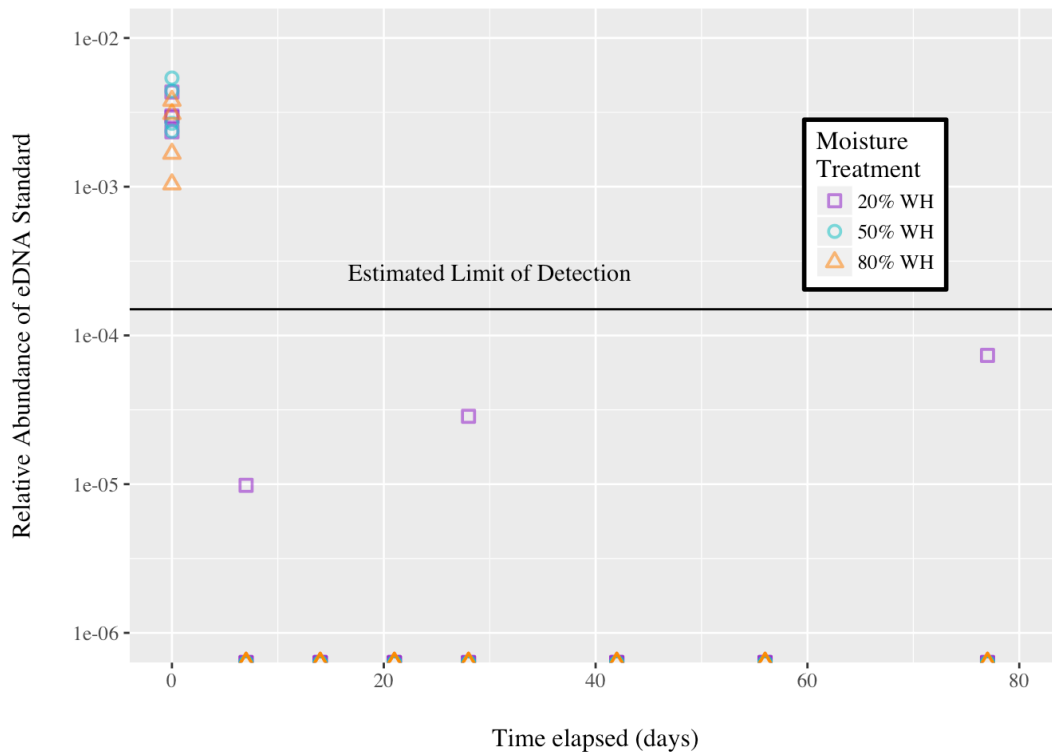


Figure 3: High-throughput sequencing results for synthetic eDNA degradation in moisture experiments. At no time were there any significant differences (one-way ANOVA, $p > 0.05$) between the 20% WHC (purple squares), 50% WHC (blue circles), or 80% WHC (orange triangles) treatments, due to the low sensitivity of high-throughput sequencing relative to qPCR.

1.4.5 Effects of temperature on eDNA dynamics

Most synthetic eDNA standard (> 99%), as measured with qPCR, was degraded within the first week regardless of temperature, but detectable eDNA standard remained stabilized in all treatments across the entire 6 week incubation period (Figure 4). At time 0, the fraction eDNA standard remaining did not differ across all 3 temperature treatments (1.00 ± 0.35 , one-way ANOVA, $p = 0.497$). Soil temperature had a significant impact on the fraction eDNA standard remaining at 7 days (one-way ANOVA: $F_{2,33} = 98.64$, $p = 1.2 \times 10^{-14}$, Tukey HSD: all $p < 7.59 \times 10^{-5}$), with the 4° C treatment having the greatest fraction of eDNA remaining ($2.9 \times 10^{-2} \pm 2.0 \times 10^{-2}$), followed by 20° C ($5.1 \times 10^{-3} \pm 1.2 \times 10^{-3}$), and 36° C ($2.2 \times 10^{-3} \pm 7.2 \times 10^{-4}$).

Soil temperature also had an impact on ultimate eDNA stabilization in soil, as estimated by model asymptotes. Stabilization of the eDNA standard was highest at 4°C (ASYM = $1 \times 10^{-2.2} \pm 1 \times 10^{-0.09}$, 95% CI), followed by the 20°C treatment (ASYM = $5.1 \times 10^{-3} \pm 1.2 \times 10^{-3}$, 95% CI) and the 36°C treatment (ASYM = $2.2 \times 10^{-3} \pm 7.2 \times 10^{-4}$, 95% CI), and this difference was significant ($p < 0.05$, as 95% CIs of model do not overlap). The 4° C treatment decay rate (LRC = -1.67 ± 0.21 , 95% CI) was significantly different from the decay rates of the 36° C treatment (LRC = -0.92 ± 0.35 , 95% CI) and 20° C treatments (LRC = -1.07 ± 0.29 , 95% CI), which were not significantly different from one another (95% CIs overlap). However, based on measurements of initial rate of decay (the disappearance of eDNA standard over the first 7 days after addition to microcosm), all three of the treatments had different initial rates of decay, with the 36° C having the fastest initial rate of decay, followed by the

20° C and the 4° C treatments (one-way ANOVA, $F_{2,33} = 98.6$, $p = 1.2 \times 10^{-14}$, Tukey HSD, all $p < 7.6 \times 10^{-5}$).

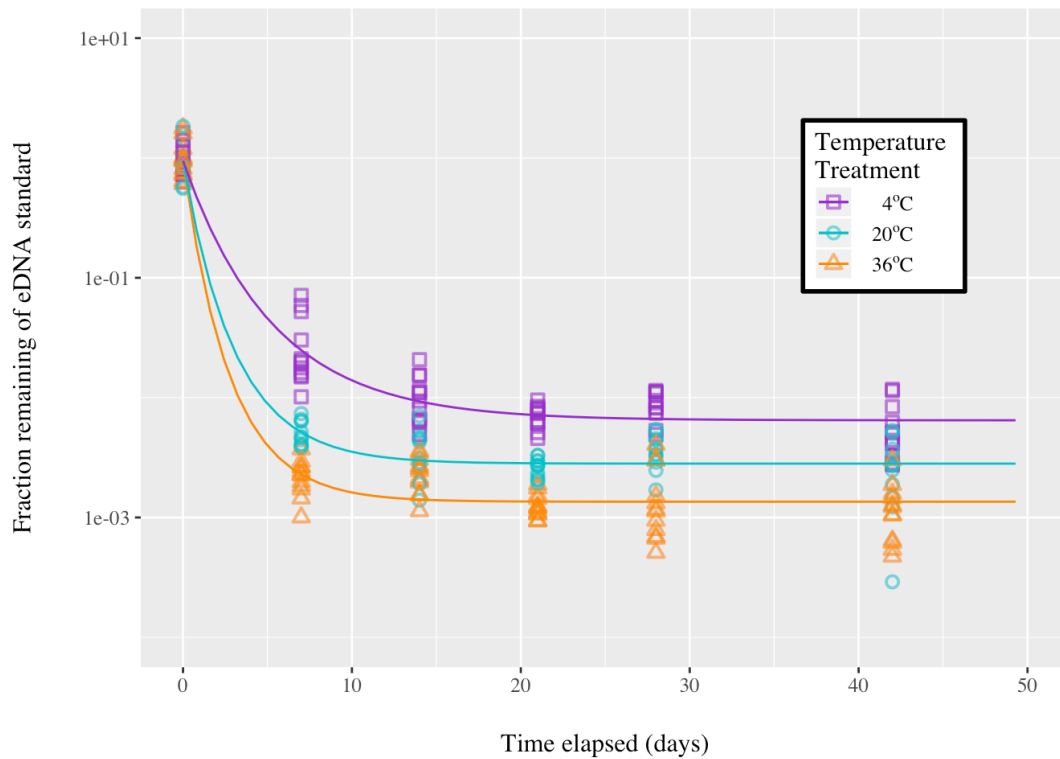


Figure 4: The qPCR data for synthetic eDNA degradation over time in 4° C (purple squares), 20° C (blue circles), and 36° C (orange triangles) treatments, with solid lines showing model predictions. The decay rate term (LRC) of the 4° C treatment was significantly different from the other temperature treatments (95% CI did not overlap). Stabilization of the eDNA standard was highest at 4° C ($ASYM = 1 \times 10^{-2.2} \pm 1 \times 10^{-0.09}$, 95% CI), followed by the 20° C treatment ($ASYM = 5.1 \times 10^{-3} \pm 1.2 \times 10^{-3}$, 95% CI) and the 36° C treatment ($ASYM = 2.2 \times 10^{-3} \pm 7.2 \times 10^{-4}$, 95% CI), and this difference was significant ($p < 0.05$, as 95% CIs of model do not overlap).

While the results from DNA sequencing of temperature experiment samples were generally consistent with those from qPCR, it was clear that sequencing had lower sensitivity than qPCR. The eDNA standard was observed to degrade rapidly in all treatments and began to fall below the limit of detection for DNA sequencing within 7

days of incubation. The standard remained detectable in the 4°C and 20°C treatments throughout the experiment, though disappeared from the 36°C treatment after week 3 (Figure 5). This result suggests greater stabilization of eDNA in the colder treatments, consistent with qPCR results, but we did not observe a significant difference in stabilization between treatments as measured by DNA sequencing (multiple one-way ANOVAs, all $p > 0.05$).

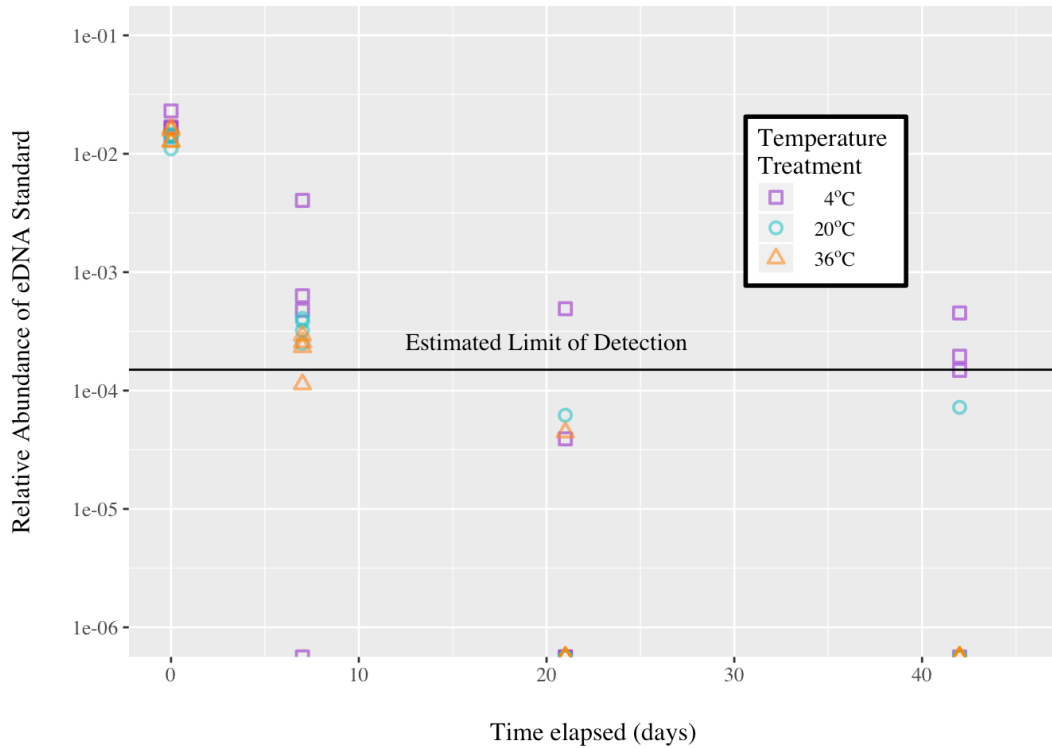


Figure 5: High-throughput sequencing results for synthetic eDNA degradation in temperature experiments. At no time were there any significant differences (one-way ANOVA, $p > 0.05$) between the 4° C (purple squares), 20° C (blue circles), or 36° C (orange triangles) treatments, due to the low sensitivity of high-throughput sequencing relative to qPCR.

1.4.6 Effects of management on eDNA dynamics:

Most synthetic eDNA standard (> 99%), as measured with qPCR, was degraded within the first week regardless of management regime, but detectable eDNA standard remained stabilized in all treatments across the entire 39-day incubation period (Figure 6). At time 0, the fraction eDNA standard remaining did not differ across all 4 management treatments (1.00 ± 0.33 , one-way ANOVA, $p = 0.99$). Soil management regime had a significant impact on the fraction eDNA standard remaining at 7 days (one-way ANOVA: $F_{3,44} = 7.29$, $p = 4.5 \times 10^{-4}$), with the NTR treatment having the greatest fraction of eDNA remaining ($5.9 \times 10^{-3} \pm 4.3 \times 10^{-3}$), which was not significantly different from the NTH treatment (Tukey HSD, $p = 0.26$), but which was significantly different from the PTH and PTR treatments (Tukey HSD, $p < 0.004$). By 14 days the two tilled treatments had diverged completely from the two no-till treatments, with the no-till treatments having a greater ($8.1 \times 10^{-3} \pm 3.0 \times 10^{-3}$) and significantly different (one-way ANOVA: $F_{1,46} = 84.6$, $p = 5.44 \times 10^{-12}$) average fraction eDNA standard remaining as compared to the tilled treatments ($2.3 \times 10^{-3} \pm 1.1 \times 10^{-3}$). This trend remained in place across the remaining 3 weeks. Differences in eDNA standard remaining were due to tillage rather than to biomass management, and there was no significant interaction between tillage and biomass (two-way ANOVAs at each time-point, tillage x biomass, all $F_{1,44} < 2.41$, all $p > 0.13$).

Soil management regime also had an impact on ultimate eDNA stabilization in soil, as estimated by model asymptotes. Stabilization of the eDNA standard was higher in no-till soils (NTR ASYM = $1 \times 10^{-2.3} \pm 1 \times 10^{-0.1}$, NTH ASYM = $1 \times 10^{-2.4} \pm 1 \times 10^{-0.1}$, 95% CIs), and significantly different (based on 95% confidence intervals)

from stabilization in the tilled soils (PTR ASYM = $1 \times 10^{-2.9} \pm 1 \times 10^{-0.1}$, PTH ASYM = $1 \times 10^{-2.8} \pm 1 \times 10^{-0.1}$, 95% CIs). The model was insensitive to differences in decay rate between tillage regimes (LRC = -0.48 ± 0.80 , 95% CI). However, based on measurements of initial rate of decay (the disappearance of eDNA standard over the first 7 days after addition to microcosm), the NTR treatment had a significantly different rate of initial decay as compared to the PTR and PTH treatments (one-way ANOVA, $F_{3,44} = 7.3$, $p = 4.5 \times 10^{-4}$, Tukey HSD, for NTH vs PTR and PTH, both $p < 0.003$).

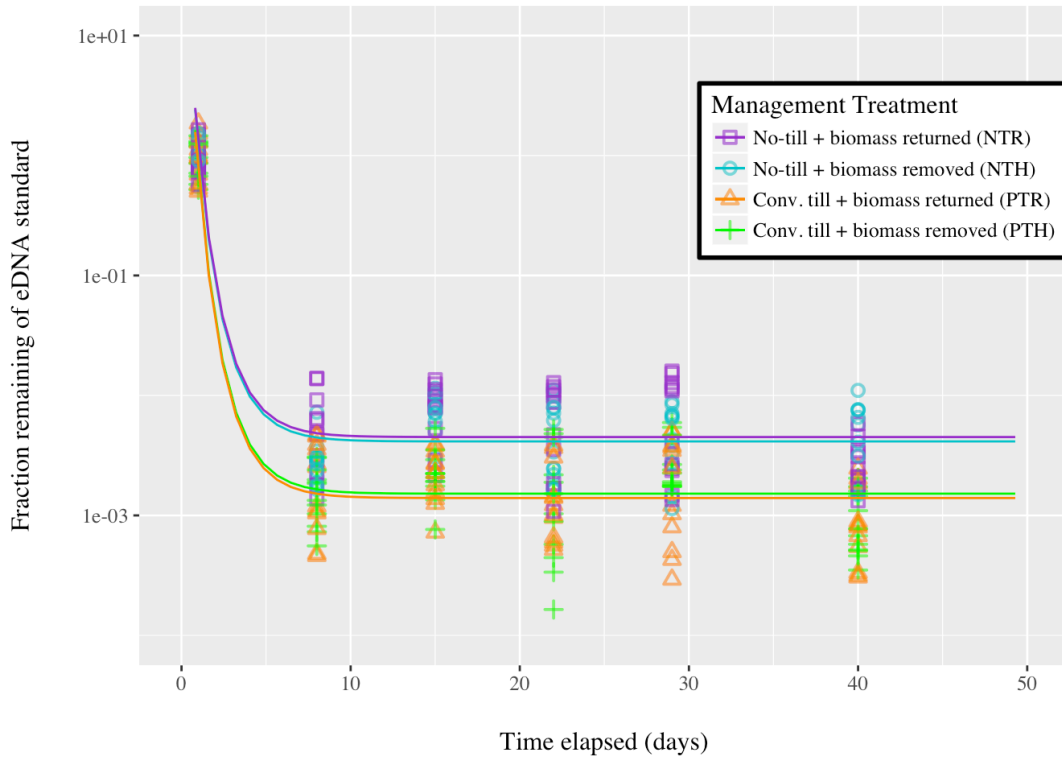


Figure 6: The qPCR data for synthetic eDNA degradation over time in NTR (purple squares), NTH (blue circles), PTR (orange triangles), and PTH (green crosses) treatments, with solid lines showing model predictions. There were no significant differences in the LRC (decay rate) term of the model between treatments (LRC 95% CIs overlapped), but there was more eDNA stabilization in the no-till treatments (NTR and NTH) as compared to the tilled treatments (PTH and PTR), a difference which was significant (95% CIs did not overlap).

While the results from DNA sequencing of management experiment samples were generally consistent with those from qPCR, it was clear that sequencing had lower sensitivity than qPCR. The eDNA standard was observed to degrade rapidly in all treatments and began to fall below the limit of detection for DNA sequencing within 7 days of incubation. The standard remained detectable in soil from all tillage regimes throughout the experiment (Figure 7). We did not observe a significant difference in stabilization between treatments as measured by DNA sequencing (multiple one-way ANOVAs, all $p > 0.05$).

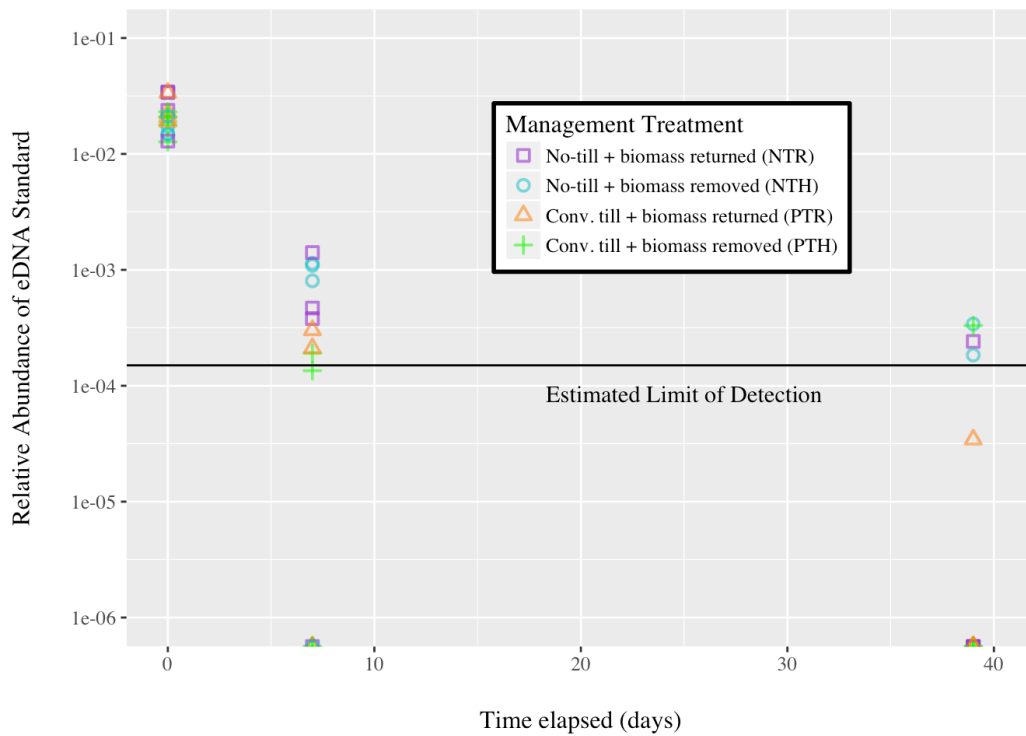


Figure 7: High-throughput sequencing results for synthetic eDNA degradation in management experiment. At no time were there any significant differences (one-way ANOVA, $p > 0.05$) between the NTR (purple squares), NTH (blue circles), PTR (orange triangles), or PTH (green crosses) treatments, due to the low sensitivity of high-throughput sequencing relative to qPCR.

1.4.7 Effects of habitat on eDNA dynamics:

Most synthetic eDNA standard (> 99%), as measured with qPCR, was degraded within the first week regardless of habitat, but detectable eDNA standard remained stabilized in all treatments across the entire 7-week incubation period (Figure 8). At time 0, the fraction eDNA standard remaining did not differ across all 3 habitat types (1.00 ± 0.45 , one-way ANOVA, $p = 0.822$). Habitat type had a significant impact on the fraction eDNA standard remaining at 7 days (one-way ANOVA: $F_{2,33} = 32.14$, $p = 1.79 \times 10^{-8}$, Tukey HSD: all $p < 6.0 \times 10^{-3}$), with the forest soils having the greatest fraction of eDNA remaining ($1.4 \times 10^{-2} \pm 2.8 \times 10^{-3}$), followed by the meadow soils ($7.3 \times 10^{-3} \pm 1.7 \times 10^{-3}$), and agriculture soils ($5.0 \times 10^{-3} \pm 2.5 \times 10^{-3}$).

Habitat type also had an impact on ultimate eDNA stabilization in soil, as estimated by model asymptotes. Stabilization of the eDNA standard was highest in meadow soils (ASYM = $1 \times 10^{-2.4} \pm 1 \times 10^{-0.10}$, 95% CI), followed by the forest soils (ASYM = $1 \times 10^{-2.6} \pm 1 \times 10^{-0.10}$, 95% CI) and the agriculture soils (ASYM = $1 \times 10^{-2.8} \pm 1 \times 10^{-0.11}$, 95% CI), and the difference between stabilization in meadow soils vs either agriculture or forest soils was significant (based on 95% confidence intervals). The forest soil decay rate (LRC = -1.80 ± 0.21) was significantly different from the decay rates of the meadow soils (LRC = -1.30 ± 0.28 , based on 95% confidence intervals), but not from the agriculture soils (LRC = -1.59 ± 0.21). However, based on measurements of initial rate of decay (the disappearance of eDNA standard over the first 7 days after addition to microcosm), all three treatments had significantly different initial rates of decay, with the agriculture having the fastest decay rate, the

meadow having an intermediate decay rate, and the forest soil have the slowest decay rate (one-way ANOVA, $F_{2,33} = 32.1$, $p = 1.8 \times 10^{-8}$, Tukey HSD, all $p < 0.006$).

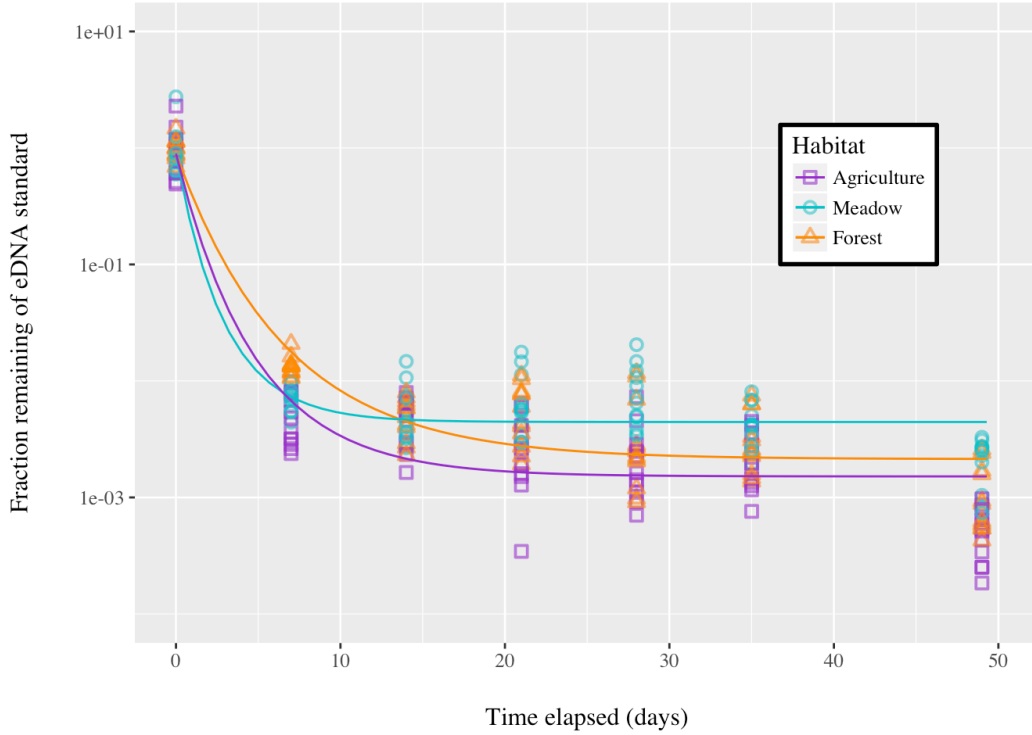


Figure 8: The qPCR data for synthetic eDNA degradation over time in agriculture (purple squares), meadow (blue circles), and forest (orange triangles) soils, with solid lines showing model predictions. The decay rate term (LRC) of the forest soils was significantly different from the meadow soils (95% CI did not overlap). Stabilization of the eDNA standard was greatest in meadow soils ($ASYM = 1 \times 10^{-2.4} \pm 1 \times 10^{-0.10}$, 95% CI), followed by forest ($ASYM = 1 \times 10^{-2.6} \pm 1 \times 10^{-0.10}$, 95% CI) and agriculture soils ($ASYM = 1 \times 10^{-2.8} \pm 1 \times 10^{-0.11}$, 95% CI), and the difference between stabilization in meadow vs either agriculture or forest soils was significant (based on 95% confidence intervals).

While the results from DNA sequencing of habitat experiment samples were generally consistent with those from qPCR, it was clear that sequencing had lower sensitivity than qPCR. The eDNA standard was observed to degrade rapidly in all treatments and began to fall below the limit of detection for DNA sequencing within 7 days of incubation. At weeks 3 and 5 no synthetic eDNA gene sequences were

detected from any habitat, and at week 7 only one sample (from forest) had any detectable eDNA sequences (Figure 9). We did not observe a significant difference in stabilization between treatments as measured by DNA sequencing (multiple one-way ANOVAs, all $p > 0.05$).

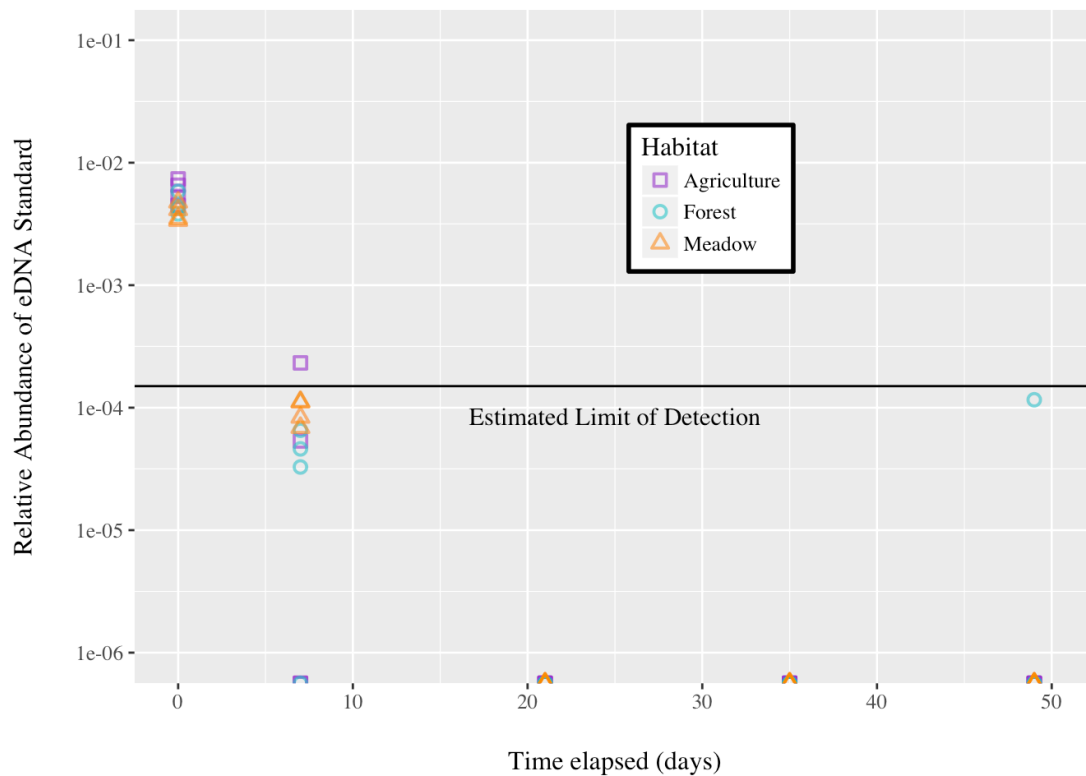


Figure 9: High-throughput sequencing results for synthetic eDNA degradation in habitat soils. At no time were there any significant differences (one-way ANOVA, $p > 0.05$) between the agriculture (purple squares), forest (blue circles), or meadow (orange triangles) soils, due to the low sensitivity of high-throughput sequencing relative to qPCR.

1.4.8 Relationships between soil C, N, pH, DNA yield and eDNA stabilization:

Across the management and habitat gradients studied (maintained at room temperature and 50% WHC), soil total C, total N, and DNA yield (ng DNA/g soil, a

proxy for soil biomass) were positively correlated (multiple Pearson's correlations, all $t_{26} > 6.9$, all $r > 0.80$, all $p < 2.6 \times 10^{-7}$). The soil pH was negatively correlated with soil C, N, and DNA yield (multiple Pearson's correlations, all $t_{26} < -5.0$, all $r < -0.70$, all $p < 3.8 \times 10^{-5}$).

There are strongly positive and significant correlations between stabilization of eDNA standard at 7 days, and soil total C (Pearson's correlation, $t_{26} = 9.5$, $r = 0.88$, $p = 6.6 \times 10^{-10}$), total N (Pearson's correlation, $t_{26} = 6.4$, $r = 0.78$, $p = 8.3 \times 10^{-7}$), and DNA yield (Pearson's correlation, $t_{26} = 4.3$, $r = 0.64$, $p = 2.1 \times 10^{-4}$). The stabilization of eDNA standard at 7 days is negatively and significantly correlated with soil pH (Pearson's correlation, $t_{26} = -4.9$, $r = -0.70$, $p = 4.6 \times 10^{-5}$). However, by the end of the management and habitat experiments (39 and 49 days, respectively), these correlations no longer exist (multiple Pearson's correlations, $-0.07 < r < 0.16$, all $p > 0.42$). There was no relationship between the amount of eDNA remaining at 7 days and the amount remaining at end of the experiment (Pearson's correlations, $r = -0.12$, $p = 0.55$). See Figure 10.

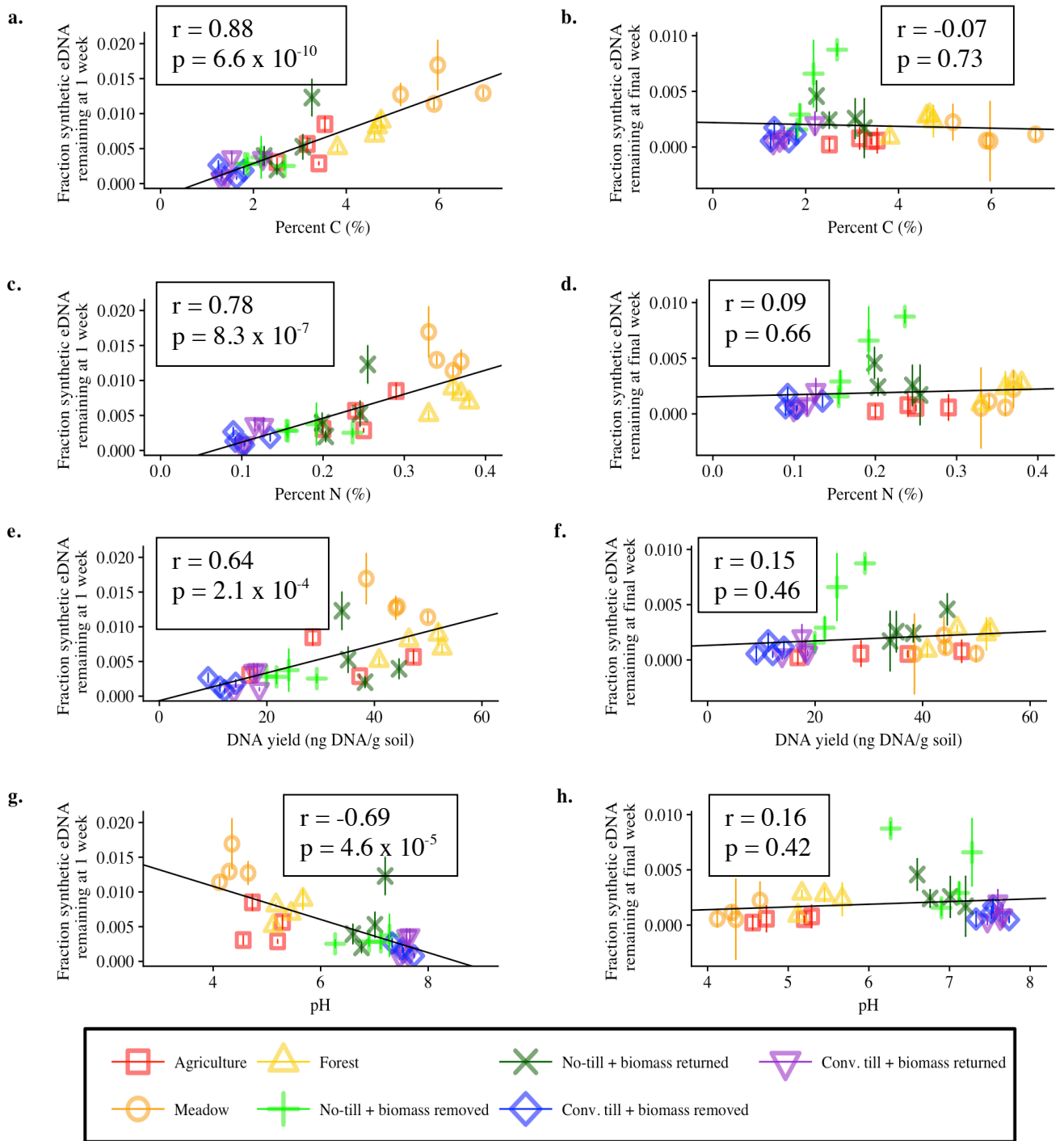


Figure 10: Relationships between eDNA stabilization and %C (a,b.), %N (c,d), DNA yield (e,f), and pH (g,h) of habitat and management soils. After 7 days there exist strongly positive and significant correlations between the stabilization of eDNA and soil C (a), N (c), and DNA yield (e), and a strongly negative and significant correlation with pH (g). After 39 or 49 days (b,d,f,h), these correlations no longer exist. Pearson's r values and p values are shown in boxes.

1.4.9 Times to Disappearance of eDNA standard from Sequencing Library:

There were differences across treatments in the time required for the eDNA standard to degrade sufficiently that it was no longer consistently detectable with high-throughput sequencing. These temporal delays in the drop below the HT-Seq LoD are shown in Figure 11, along with estimates of the error range, based on the standard deviation of the high-throughput sequencing LoD.

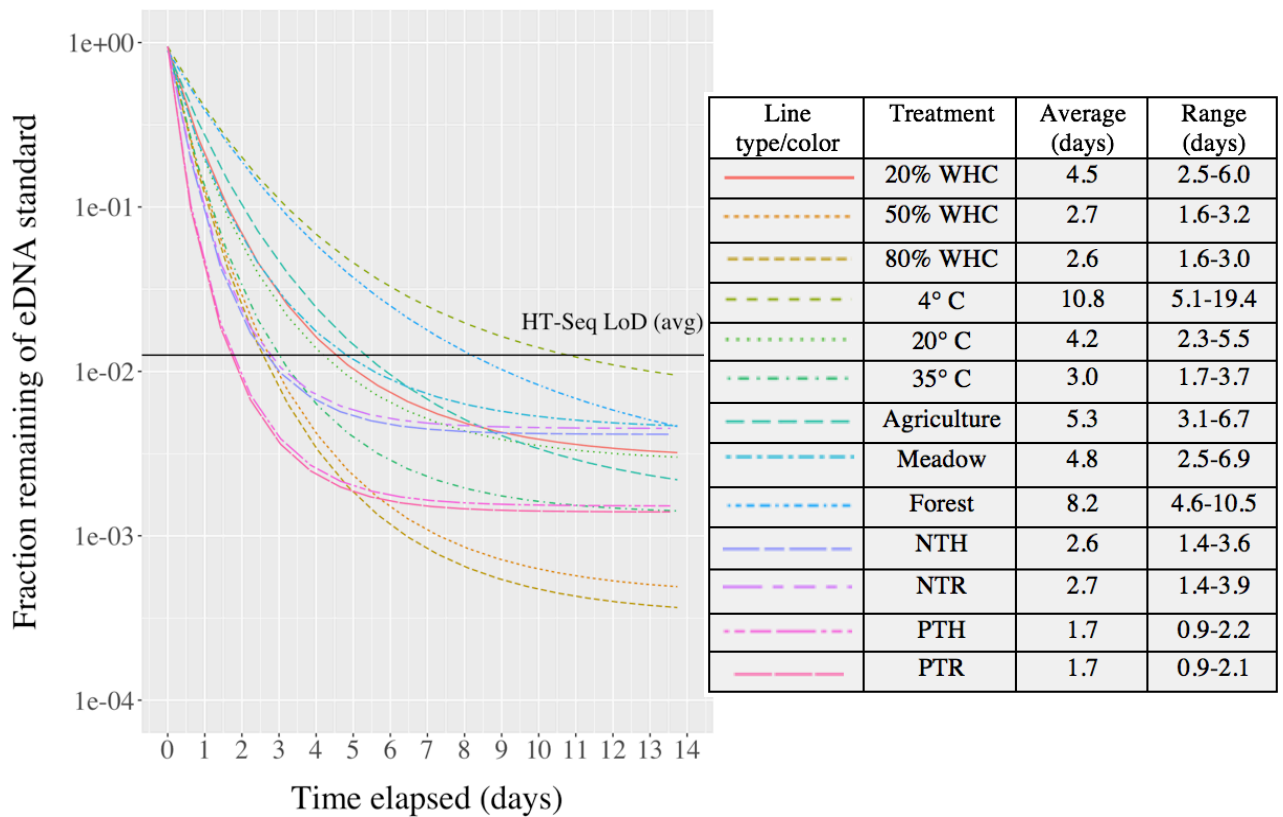


Figure 11: Treatment-driven temporal delays in the degradation of the eDNA standard below the high-throughput sequencing LoD, with estimates of range based on the standard deviation of the estimated high-throughput sequencing LoD.

1.5 Discussion:

We treated soils with a synthetic eDNA standard as part of a controlled laboratory experiment to test the effects of soil moisture, temperature, agricultural management (till vs no-till, and biomass removed vs returned), and habitat type on the degradation/stabilization dynamics of eDNA in soils. Across treatments, eDNA degraded very rapidly, being reduced by >99% by 7 days, and remained detectable throughout the experiment (39-80 days). Moisture, temperature, tillage regime, and habitat significantly impacted eDNA initial degradation and ultimate stabilization, with the most stabilization occurring at low moisture, low temperature, in soils managed without tillage, and in meadow soils (see Figure 12 for a theoretical framework). It is likely very important to recognize the differential degrees of bias introduced by eDNA under diverse circumstances, and to respond with appropriate experimental design and analytical approaches. How appropriately we can respond to the potential for eDNA-driven bias, however, will depend on the quality and quantity of our knowledge surrounding the issue.

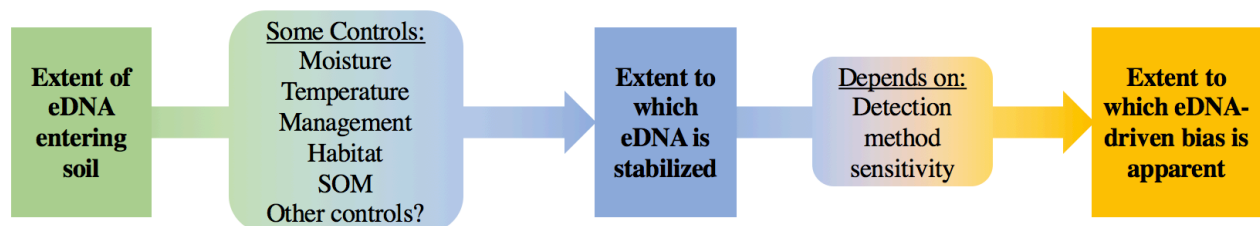


Figure 12: Theoretical framework of the effects of soil condition and laboratory method gradients on eDNA-driven bias in estimates of microbial community composition in soils. Moisture, temperature, and tillage intensity are all positively correlated with greater eDNA degradation, while detection method sensitivity is positively correlated with the extent to which eDNA-driven bias is apparent in community estimates.

The results obtained here have furthered our understanding of eDNA dynamics in soils, and should be taken into consideration during any measurements of soil microbial community composition. It is clear that soil moisture, temperature, habitat, and tillage regime impact eDNA degradation/stabilization dynamics in soil, and that the effects thereof persist across several months, if not longer, depending on detection method. Importantly, while differences in eDNA degradation dynamics were detectable with qPCR, the more sensitive method of those used here, differences were not detectable in any case with high-throughput sequencing. This implies that the extent to which eDNA-driven bias is apparent depends on the sensitivity of the detection method (Figure 12), and that high-throughput community sequencing, even within a few days of eDNA entering soil, might not be sensitive enough to detect sufficient eDNA to result in bias in the apparent community structure. Specifically, the duration of eDNA-driven bias apparent with high-throughput sequencing in this study was estimated to range from 0.9 - 19.4 days. This would indicate that any study hoping to measure changes in microbial community structure at shorter intervals than the amount of time it takes for eDNA sequences to drop below the limit of detection for that particular method might encounter bias in estimates of community composition like those found in other studies (Carini et al. 2017).

Our finding that higher moisture and temperature result in greater degradation and reduced stabilization of eDNA in soils is consistent with previous findings (Widmer et al. 1996). These relationships are likely the result of reduced activity of extracellular DNases at lower temperatures, and both/either diffusion limitation and/or a reduction in microbial activity under low moisture conditions. Soils that are cold

and/or dry are more likely to have greater stabilization of eDNA, and therefore estimates of microbial community structure from these soils may be subject to greater eDNA-driven bias.

Our findings regarding the relationships between eDNA stabilization and SOM are somewhat more surprising. Because eDNA degradation is largely driven by soil microbes, we might have expected the higher microbial biomass in the no-till plots to result in greater eDNA degradation than in the tilled plots. In contrast, what we observe is that no-till management results in significantly greater eDNA stabilization than conventional tillage. Soil microbial biomass, C, and N all are strongly correlated, and together can be taken as a proxy for soil organic matter (SOM). Greater SOM is correlated with greater eDNA remaining after 7 days. There is not, however, any correlation between SOM content and degree of eDNA stabilization by the end of the experiment (6-7 weeks). This indicates that while SOM plays a role in the initial degradation rate of eDNA entering a soil system, that ultimately SOM is not likely to change longer-term eDNA stabilization capacity of a soil. Clearly there are controls other than SOM on the extent to which eDNA is stabilized in soils. We propose that soil structure (specifically in regards to aggregates), the specific physicochemical makeup of soil components, and the structure of the microbial community itself may also control the extent to which eDNA is stabilized in soils, though future studies are needed to confirm this.

Similarly to Carini et al. (2017), we found relationships between eDNA stability and edaphic characteristics such as pH and SOM. In contrast to Carini et al. (2017), however, we found that eDNA-driven bias is not likely in most soils, with

eDNA being degraded very shortly after entering the system. Lennon et al. (2017) concluded that eDNA, even under conditions favoring protection, is still unlikely to generate bias in estimates of microbial diversity. Our findings here support that conclusion, specifically because even when degradation of eDNA was greatly reduced (under dry or cold conditions, for example), community sequence data were not able to consistently detect the eDNA standard after one week. This would suggest that even though eDNA may still be present in the soil over long time spans, its degradation is rapid enough that even after only a few days, it is unlikely to bias community estimates when the detection method in use is high-throughput sequencing.

While the current approach resulted in a controlled ability to measure eDNA degradation/stabilization in microcosms, in natural systems eDNA is neither pure nor consistent in its composition. The source of eDNA, as well as its composition, influences stabilization/degradation dynamics, though the mechanisms and patterns are not well understood (Gulden et al. 2005; Pietramellara et al. 2009). There is evidence that eDNA from unpurified cell lysate may experience different fates in soils as compared to purified eDNA (Nielsen et al. 2000), that DNA fragments of different lengths experience different fates (Ogram et al. 1988; Ogram et al. 1994; Pietramellara et al. 2009), and that G + C content of eDNA influences degradation dynamics (Dell'Anno et al. 2002; Vuillemin et al. 2017). Our eDNA fragment was of a consistent length (~500 bp), a consistent sequence, and a consistent G+C content (36%), and may not have allowed us to capture some subtleties of eDNA degradation/stabilization in nature.

Importantly, our experiments only ranged across 39-80 days. Compared to natural systems, this time frame may not have been sufficient to capture the long-term dynamics of eDNA in soils. The current experiment also lacked temporal resolution within the first week after eDNA addition, which resulted in an inability in many cases to distinguish the impacts of treatment on modeled initial eDNA degradation rates. The current study was designed to measure the disappearance of a single addition of eDNA to a soil, but in natural systems eDNA is constantly being added to and degraded from the soil. We did not measure the results of repeated eDNA addition to the soil, and as such cannot speak to the presence or absence of an eDNA “reservoir” in the soil. For example, do repeated additions of eDNA “fill up the reservoir” and result in increasingly more eDNA persistence? Is there a threshold beyond which no further eDNA can be stabilized in a potential eDNA “reservoir”? Our results indicate that if such reservoirs are the mechanism of eDNA stabilization in soils, different environmental and soil conditions (moisture, temperature, tillage) would result in different contributions to said reservoirs.

Future studies should examine eDNA dynamics in soils when DNA additions are more representative of reality, i.e. occur repeatedly or continually. Efforts to use eDNA of varying condition and composition (i.e. length, G+C content, biological source, purity), more representative of the wide array of eDNA present in natural systems, will also be important in future studies. Additionally, we should pursue finer resolution of eDNA degradation within the first hours or days after addition to soils, depending on the importance of smaller timescales to a given study. The relationship between G+C content and eDNA stability has been observed, but has not been tested

experimentally (Dell’Anno et al. 2002; Vuillemin et al. 2017). Future studies should look to experimentally test this relationship, given that the G+C content of bacterial genomes is related to phylogeny (Gupta 2000), so differential eDNA degradation due to G+C content differences may result indirectly in over- or under-representation of some phyla in community analyses when eDNA is not taken into consideration. This is consistent with the conclusions of Lennon et al. that eDNA-related bias in community composition measurements is likely when the species distributions of the iDNA and eDNA pools are distinct (Lennon et al. 2017). Finally, consideration of the relationships between soil structure and eDNA dynamics should be explored, including special attention to aggregation, pore size, and spatial heterogeneity.

As our ability to deeply sequence DNA from soils improves, we must bear in mind the potential impacts of eDNA on our estimates of microbial community. It is widely accepted that eDNA exists in substantial quantities in soils, but we lack a nuanced understanding of the impacts eDNA on community estimates. The current study demonstrates that eDNA degradation and stabilization dynamics differ across gradients of soil condition, and that even while some eDNA may be indefinitely stabilized in soils, we found little evidence that this stabilized eDNA is likely to introduce bias to community sequence data. With the present work, we add to the conversation surrounding the impacts of eDNA on soil microbial community structure estimates, and it is our hope that future studies will further elucidate the details of these complicated and fascinating interactions.

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CHAPTER 2

SOIL EXTRACELLULAR DNA: ITS BEHAVIOR IN SOILS AND IMPACTS ON ESTIMATES OF MICROBIAL COMMUNITY STRUCTURE

2.1 Abstract

Extracellular DNA (eDNA) may arise from either cell lysis or DNA secretion. Soils may accumulate substantial quantities of eDNA, which may persist for long periods of time and experience a variety of fates. Questions about soil eDNA have recently risen to the forefront of molecular biology and microbial ecology because of the potential of eDNA to bias sequence-based estimates of microbial community composition. Here we review the impacts of eDNA on microbial community estimates in soils, including evidence both for and against eDNA-driven bias in community estimates. We discuss the influence of both intrinsic properties and external soil conditions on eDNA stabilization/degradation dynamics, and the relationships between eDNA dynamics and eDNA-driven bias to community structure estimates. In order to accurately characterize microbial communities using culture-independent methods, it is imperative that we determine the extent to which eDNA introduces bias to community estimates, and understand under what circumstances such bias is most likely to arise.

2.2 *Introduction*

Extracellular DNA (eDNA) is any single- or double-stranded DNA molecule not contained within a cell membrane. In soils, eDNA can reach levels of 2ug per gram of soil (Niemeyer and Gessler 2002), constitutes up to 80% of the total extractable DNA pool (Carini et al. 2017; Lennon et al. 2017), and can persist for weeks or years (reviewed in Kaare M. Nielsen et al. 2007; Levy-Booth et al. 2007; Pietramellara et al. 2009), and even for millennia (Perkins 2003; Willerslev et al. 2003; Slon et al. 2017). This eDNA has the potential to introduce bias to estimates of microbial community structure. Such bias might interfere with our understanding of the structure and function of microbial communities, especially when temporal changes in those communities are important. When using DNA-based community measurement techniques, specifically environmental gene sequencing, there exists the implicit assumption that each DNA sequence found derives directly from a living cell. That is to say, we assume that the cell from which the sequence came was alive in the sample, and was lysed only when its DNA was extracted as part of the sequencing workflow. In reality, eDNA violates this assumption. Because eDNA can be stabilized on soil components (Goring and Bartholomew 1952; Ogram et al. 1988; Ogram et al. 1994) and can persist for long periods of time (Levy-Booth et al. 2007; Pietramellara et al. 2009), eDNA has the potential to remain sequenceable long after the cell from which it derived has died (See Chapter 1; and Widmer et al. 1996). Sequences from eDNA are indistinguishable from those of intracellular DNA (iDNA), and as such may be creating the illusion of long-dead cells still being present in soils (Figure 1). Because eDNA may be systematically introducing bias to sequence data, and because

there exists no *post hoc* process to correct for this bias, we have no way to determine the extent to which sequence data are or are not representative of reality. A variety of methods have been used in attempts to eliminate or segregate eDNA in soil samples (Dell'Anno et al. 2002; Taberlet et al. 2012; Villarreal et al. 2013; Alawi et al. 2014), but none of these methods is currently widely used in high-throughput sequencing protocols.

DNA sequencing as a method of data collection has become increasingly popular in research of all types, especially with the advent of fast, low-cost high-throughput sequencing technologies. Microbial ecologists, in particular, have taken to high-throughput sequencing as a powerful tool to estimate microbial community structure. Because many environmental bacteria are difficult (or impossible) to culture or to identify based on morphology or physiology, sequencing provides a method of taxonomic identification that relies solely on genetic information, requiring neither culturing nor microscopy. Sequencing of taxonomic markers, such as the bacterial 16S rRNA gene or the fungal ITS gene, allows environmental microbiologists to identify microbes in a sample, and to arrive at estimates of taxonomic distribution and potential function.

This review discusses current knowledge surrounding two questions: (1) What are the behaviors of eDNA in soil and how do those behaviors relate to eDNA-driven bias? and (2) To what extent does eDNA introduce bias to estimates of soil microbial community structure?

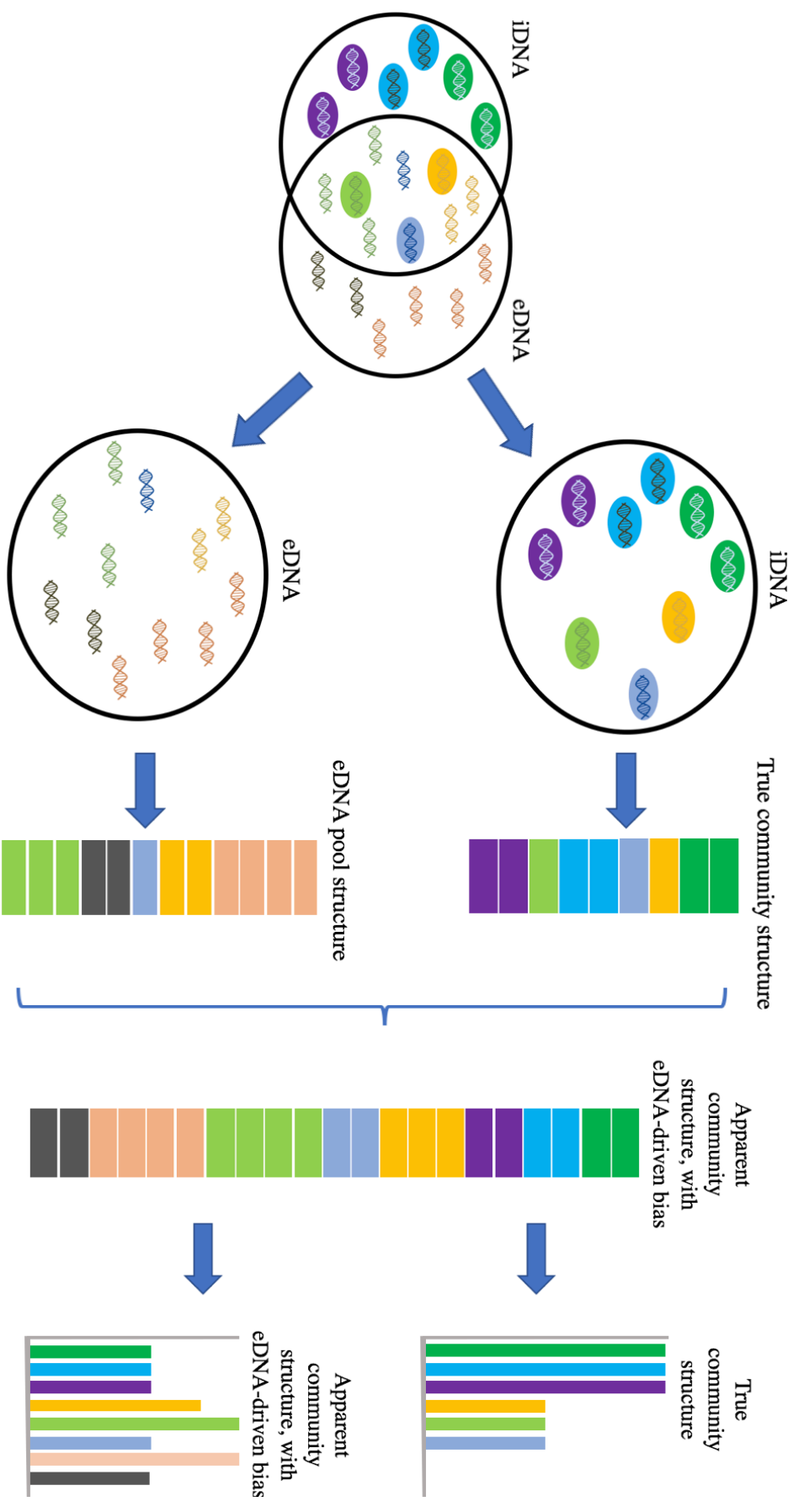


Figure 1: The process by which eDNA may introduce bias to estimates of microbial community structure. Sequences from long-dead cells (eDNA) may remain detectable in the soil, and may not be representative of the living microbial community.

2.3 Behavior of eDNA in Soils

Understanding the pathways through which eDNA is introduced to, is stabilized within, or is removed from the soil is key to grasping the significance of eDNA in microbial ecology, nutrient cycling, and research methodology.

2.3.1 Entrance of eDNA to the soil

In soils, a major source of eDNA is microbial cell death and lysis as a result of predation, autolysis, infection, or necrosis, though active microbial secretion of eDNA into the environment is also common (Nielsen et al. 2007; Vorkapic et al. 2016; Ibáñez de Aldecoa et al. 2017). Both bacterial and eukaryotic biofilms often contain eDNA as part of their extracellular matrix, where the eDNA plays important roles in biofilm organization, structure, persistence, and resistance (reviewed in Vorkapic et al. 2016). Any biotically-derived material, such as sloughed plant roots, decaying biomass, or animal scat can release eDNA into soils as the material degrades. Because the soil is an inherently heterogeneous environment that experiences a vast array of environmental conditions and levels of disturbance, and because the soil is subject to a wide variety of potential eDNA sources, determining the quantities, qualities, and fates of eDNA in the soil is quite challenging.

2.3.2 Fates of eDNA in soils

Extracellular DNA in the soil may experience a variety of fates (Figure 2), namely (1) degradation by DNases, (2) stabilization on soil components, or (3) incorporation into another bacterial genome through horizontal gene transfer (Levy-

Booth et al. 2007). Because DNA is a rich source of both nitrogen (N) and phosphorus (P), nutrients frequently limited in soil systems (Walker and Syers 1976; Vitousek and Howarth 1991; Elser et al. 2007), bacteria employ DNases to assist in the utilization of nucleic acids as a nutrient source (Blum et al. 1997). Prokaryotes ranging from the very familiar *Escherichia coli* to the exotic halophilic archaeon *Haloferax volcanii* have demonstrated the ability to degrade eDNA as a nutrient source (Finkel and Kolter 2001; Chimileski et al. 2014). Interestingly, *E. coli* subsisted equally well on both endogenous and exogenous eDNA, while *H. volcanii* could subsist on both but selectively metabolized endogenous DNA (Finkel and Kolter 2001; Chimileski et al. 2014). Presumably some microbes are better adapted to degrade and consume eDNA than are others, or are less specific in the eDNA they can metabolize. In addition, different microbes may utilize distinct methods of eDNA mineralization. Specifically, it is widely accepted that microbial DNases play a key role in eDNA degradation, but there is evidence that *E. coli* can directly uptake long DNA strands for metabolism (Finkel and Kolter 2001).

Because nucleotides are energetically expensive to synthesize *de novo*, eDNA may also be broken down enzymatically into its component nucleotides and re-assembled into DNA within the microbial cell (Levy-Booth et al. 2007). The addition of eDNA to a soil system results in increases in microbial growth (Blum et al. 1997) and shifts in community structure as bacterial taxa respond differentially to the nutrient input (Morrissey et al. 2015). Morrissey et al. (2015) saw that certain phyla

increased after being fed eDNA, specifically a number of unclassified genera as well as members of the *Arthobacter*, *Nocardioides*, and *Flavisobacter*.

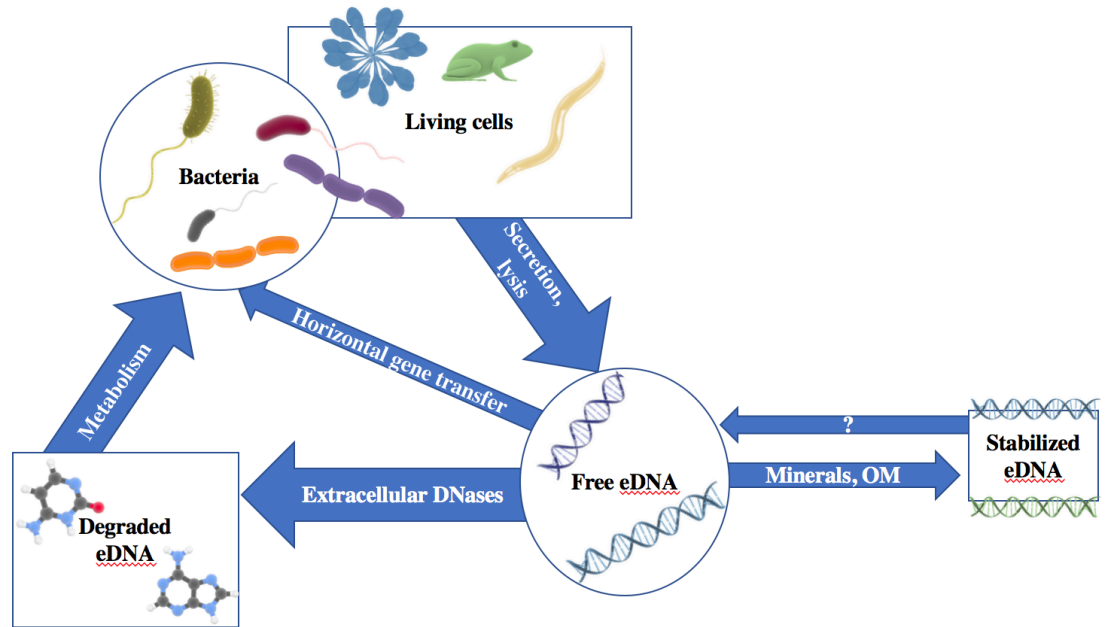


Figure 2: eDNA cycling in the environment, showing the three fates of eDNA: degradation, stabilization, or uptake via HGT. Figure based in large part on the review by Levy-Booth et al. (2007).

Stabilization on soil minerals and organic matter can protect eDNA from biotic degradation (Levy-Booth et al. 2007). DNA is strongly bound by clays (Goring and Bartholomew 1952; Greaves and Wilson 1969) and by “humic” substances (Crecchio and Stotzky 1998; Saeki et al. 2011), and is held to a lesser extent by sands (Lorenz and Wackernagel 1987). Morrissey et al. (2015) found that the effects of eDNA addition on microbial community structure varied with different clay components present in the soils, leading the authors to conclude that clay minerals may affect eDNA-driven community changes via their role in regulating the availability of

nutrients with organo-mineral complexation. Additionally, there is a negative relationship between soil organic matter (SOM) content and initial eDNA degradation rate, but no relationship exists between SOM and the longer-term (6-7 weeks) extent of eDNA stabilization (See Chapter 1).

Finally, intact eDNA may be incorporated into cellular genomes through horizontal gene transfer (HGT), contributing to genetic adaptation in soil microbial communities (Lorenz and Wackernagel 1994). The ability of bacteria to be transformed in soils is dependent on both moisture and nutrient availability (Nielsen et al. 1997). Additionally, there is evidence that while stabilization on soil components may protect eDNA from enzymatic degradation (Lorenz and Wackernagel 1987), that such stabilized eDNA may still be available for uptake through HGT (Crecchio and Stotzky 1998). Of special concern in HGT is DNA derived from genetically modified organisms (GMOs). While this review will delve into detail regarding neither HGT nor GMO DNA, nor the relationship between the two, other works and reviews are suggested, namely those by Lorenz and Wackernagel (1994), Widmer et al. (1996), Nielsen et al. (1997), Crecchio and Stotzky (1998), Nielsen et al. (2000), Gulden et al. (2005), Gebhard and Smalla (2006), Levy-Booth et al. (2007), Nielsen et al. (2007), Pote et al. (2010), and Vorkapic et al. (2016).

2.3.4 Factors affecting eDNA degradation/stabilization dynamics

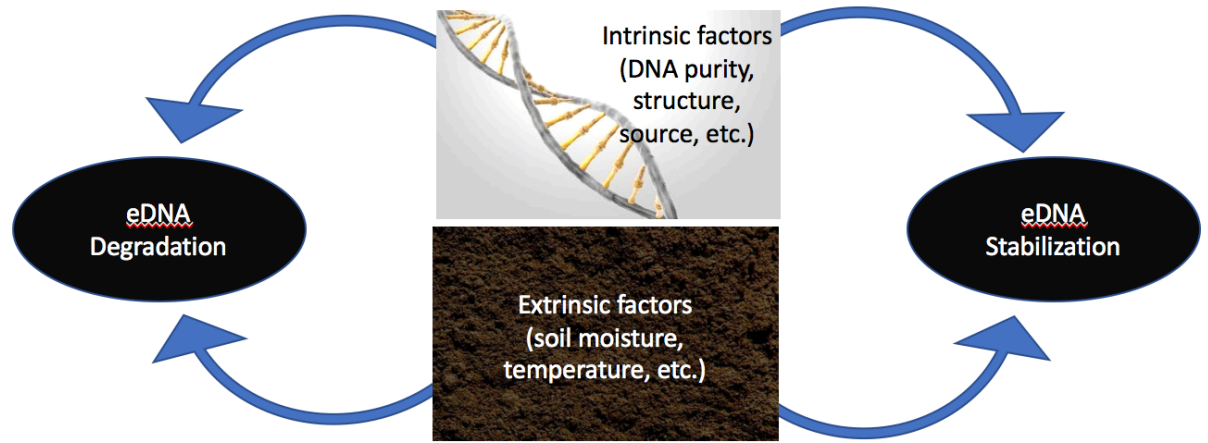
The extent to which eDNA introduces bias to estimates of microbial community structure is related to the degradation/stabilization dynamics of eDNA. On one extreme, if all eDNA were to instantly degrade, no eDNA bias would ever be

present, but if all eDNA were to be stabilized forever, the sequencing signal from iDNA would be lost in the signals from all previous communities present only as eDNA. Neither of these extreme examples reflect reality, but it is widely accepted that some portion of the eDNA introduced to soil will be stabilized. Differential stabilization across gradients of conditions may result in differential eDNA-driven bias in estimates of community structure. Therefore, understanding the degradation/stabilization dynamics of eDNA in soils is likely one of the keys to understanding the extent to which estimates of community structure are being impacted by eDNA.

The degradation/stabilization dynamics of eDNA are controlled by both intrinsic and extrinsic properties (Table 1). The biological source of eDNA influences its rate of degradation (Gulden et al. 2005) and sorption dynamics (Pietramellara et al. 2009). The G+C content of the DNA also influences persistence, with higher G+C resulting in generally more stability (Dell'Anno et al. 2002; Vuillemin et al. 2017). When eDNA is present in soils as an unpurified cell lysate, it is available for uptake through HGT for longer than purified genomic eDNA (Nielsen et al. 2000). Molecular weight (MW) plays a role in eDNA adsorption, though studies are conflicted. Ogram et al. (1988) found that high MW eDNA sorbed more readily than low MW eDNA, but later found that low MW eDNA sorbed preferentially (Ogram et al. 1994). Pietramellara et al. (2009) make the case that low MW eDNA sorbs in greater quantity, but that higher MW eDNA sorbs more strongly. There is also evidence that DNA form influences sorption, as the adsorption dynamics of supercoiled plasmid DNA differ from those of linear DNA on clays (Poly et al. 2000).

The extrinsic soil environment is complex and heterogeneous, which renders the determination of eDNA behavior in soils quite difficult. It is widely accepted that soil minerals can play a large role in stabilizing eDNA through adsorption (Levy-Booth et al. 2007; Pietramellara et al. 2009), with clays adsorbing eDNA quite strongly (Greaves and Wilson 1969; Ogram et al. 1988), and sands adsorbing eDNA somewhat, but to a lesser extent (Lorenz and Wackernagel 1987; Ogram et al. 1994). More recent studies indicate that while soil minerals play an important role in eDNA adsorption, laboratory treatments may overestimate soil adsorption capacity (Gardner and Gunsch 2017). Soil organic matter (SOM), such as “humic” substances, plays a more ambivalent role in eDNA adsorption, with some studies finding no relationship between total sorbed DNA and SOM content (Ogram et al. 1988) and others finding that humic substances adsorb substantial quantities of eDNA (Crecchio and Stotzky 1998; Saeki et al. 2011). There is also evidence that SOM is significantly and positively correlated with eDNA stabilization within the first week after eDNA enters the system, but that by 6-7 weeks there is no longer any correlation between SOM and eDNA stabilization (See Chapter 1). Additionally, pH plays an important role in the adsorption of eDNA to surfaces, with low pH electrostatically increasing DNA’s ability to sorb to negatively charged clays (Levy-Booth et al. 2007). Other electrostatics-related edaphic properties, such as cation exchange capacity, exchangeable ions, and electrical conductivity, are also correlated to eDNA content in soils (Carini et al. 2017).

Table 1: Extrinsic and intrinsic controls on the fates and behaviors of eDNA in soils.



Control (extrinsic)	Relationship with eDNA dynamics	System tested	Sources
Moisture	Higher soil moisture results in greater eDNA degradation	nonsterile soil microcosms	(See Chapter 1)
Moisture	Higher soil moisture results in greater eDNA degradation	nonsterile soil microcosms	(Widmer et al. 1996)
Temperature	Higher temperature results in greater eDNA degradation	nonsterile soil microcosms	(See Chapter 1)
Temperature	Higher temperature results in greater eDNA degradation	nonsterile soil microcosms	(Widmer et al. 1996)
Temperature	Higher temperature is correlated with greater eDNA degradation	plant growth cylinder leachate	(Gulden et al. 2005)
Tillage regime	More intensive tillage leads to greater eDNA degradation	nonsterile soil microcosms	(See Chapter 1)
Habitat	Unclear; forest soil experienced greatest initial eDNA decay, but meadow soils had the most stabilized eDNA at 7 weeks	nonsterile soil microcosms	(See Chapter 1)
Fertilizer regime	Greater fertilization results in less eDNA stabilization	agricultural soils	(Niemeyer and Gessler 2002)

Clay	Clays readily sorb eDNA	sterile batch slurry sorption isotherms	(Ogram et al. 1988)
Clay	Clays readily sorb eDNA	montmorillonite sorption isotherm	(Greaves and Wilson 1969)
SOM/humics	SOM was negatively correlated with initial eDNA degradation, but had no relationship with longer-term stabilization	nonsterile soil microcosms	(See Chapter 1)
SOM/humics	Humics were unimportant in eDNA adsorption in soils	sterile batch soil slurry sorption isotherms	(Ogram et al. 1988)
SOM/humics	Humics readily sorb eDNA	sterile sorption isotherms with purified humics	(Crecchio and Stotzky 1998)
SOM/humics	Humics readily sorb eDNA	sterile sorption isotherms with purified humics	(Saeki et al. 2011)
pH	pH is negatively correlated with eDNA stabilization	environmental soil surveys	(Carini et al. 2017)
pH	pH was positively correlated with initial eDNA degradation, but had no relationship with longer-term stabilization	nonsterile soil microcosms	(See Chapter 1)
Soil ions (CEC, exchangeable bases, EC)	Soil ions were negatively correlated with eDNA stabilization	environmental soil surveys	(Carini et al. 2017)
Soil ions (CEC, exchangeable bases, EC)	Addition of electrolytes increased eDNA adsorption, regardless of pH	montmorillonite sorption isotherm	(Greaves and Wilson 1969)
Control (intrinsic)	Directionality	System tested	Sources
biological source of eDNA	influences rate of degradation	plant growth cylinder leachate	(Gulden et al. 2005)

biological source of eDNA	influences sorption dynamics	montmorillonite sorption isotherm	unpublished data, cited in (Pietramellara et al. 2009)
G+C content	higher G + C leads to greater stability of eDNA	marine sediment cores	(Dell'Anno et al. 2002)
G+C content	higher G + C leads to greater stability of eDNA	lacustrine sediment cores	(Vuillemin et al. 2017)
purity	Unpurified cell lysate DNA remains available for uptake to HGT longer than purified DNA	sterile and nonsterile soil microcosms	(Nielsen et al. 2000)
molecular weight	high MW sorbs more readily	sterile batch slurry sorption isotherms	(Ogram et al. 1988)
molecular weight	low MW sorbs preferentially	sterile batch slurry sorption isotherms	(Ogram et al. 1994)
DNA structure (plasmid vs linear)	eDNA sorption dynamics differ, but overall stability is similar between eDNA structural forms	sterile mineral sorption isotherm	(Poly et al. 2000)

Decreasing soil temperature has been shown to decrease eDNA degradation, consistent with the concept that the vast majority of eDNA degradation is driven by enzymatic reactions (Widmer et al. 1996). At 4° C eDNA degradation is slower than at 20° C or 36° C, at which temperatures degradation is similar (Widmer et al. 1996). Decreased moisture content of the soil also results in decreased eDNA degradation versus higher moisture (Widmer et al. 1996). In one study, temperature differences (4° C vs 36° C) were estimated to introduce a nearly 10-day difference in the duration of eDNA-driven bias in apparent community structure measured with high-throughput

sequencing (See Chapter 1). The concentration of eDNA in soils also appears to be negatively correlated with fertilizer application (Niemeyer and Gessler 2002).

2.4 *eDNA-driven bias in estimates of microbial community*

Now that we have explored the intrinsic and extrinsic factors affecting the behavior of eDNA in soils, we can return to our second question. To what extent does eDNA introduce bias to estimates of soil microbial community structure? There is evidence both for and against eDNA-driven bias in estimates of soil microbial community structure. In either case, studying eDNA in soils is a challenging affair, fraught with obstacles and pitfalls (Figure 3).

Obstacles to the study of eDNA in soils:

- Semantic:
 - We lack consensus in definition of “eDNA” (intact but dead cells? Viruses?)
- Methodological:
 - DNA-based methods are imperfect (PCR bias, primer bias, chimeras, bias in analysis, etc.)
 - Viability PCR has drawbacks (re-wetting soils may artificially lyse cells, dyes may be toxic, etc.)
 - Difficult to extract all eDNA without lysing cells
 - DNases degrade free and adsorbed eDNA differentially
 - Culturing is not representative of community
- Material:
 - Soil aggregates may occlude eDNA
 - Soil components may differentially sorb eDNA
 - Biofilm matrix eDNA may be recalcitrant
 - Soil is opaque (interferes with microscopy)

Figure 3: Semantic, methodological, and material obstacles to the study of eDNA in soils.

2.4.1 Evidence that eDNA is introducing significant bias

Some studies have determined that eDNA plays havoc with sequence-based community analysis. For example, Carini, et al. (2017) found that, on average, eDNA comprised $40.7 \pm 3.75\%$ of the total amplifiable microbial 16S genes from 31 soils, and had profound effects on apparent bacterial community structure. Specifically, they found that removal of eDNA resulted in an average reduction in prokaryotic richness of $13.9 \pm 1.20\%$, and that in some soils the reduction in richness was as much as 55% (Carini et al. 2017). Additionally, the composition of prokaryotic communities in all 31 soils tested were significantly different (q value ≤ 0.05) after removal of eDNA, but in several soils, eDNA removal did not result in a significant alteration of apparent community diversity (Carini et al. 2017). Fungal eDNA may also introduce bias into estimates of soil community, as Carini et al. (2017) found that most fungal communities tested were significantly different between samples with and without eDNA removal. Importantly, they found that certain edaphic characteristics related to electrostatic interactions were significant predictors of eDNA content $\geq 20\%$ (Carini et al. 2017). Relationships between edaphic characteristics (pH, %C, and %N) and short-term eDNA stabilization have also been found in other studies, though there was no correlation between longer-term eDNA stabilization and edaphic characteristics (See Chapter 1).

The method used by Carini, et al. (2017) relied on the DNA-intercalating, photosensitive dye propidium monoazide (PMA), which was applied to soil samples to render eDNA un-amplifiable in subsequent PCR steps. PMA cannot penetrate intact cell walls, and therefore selectively excludes only eDNA from downstream analyses

(Emerson et al. 2017). Because of its selectivity, PMA methods have been proposed for public health applications as a way to amplify only DNA from viable/intact cells, useful in confirming that disinfection of a system was effective (Taylor et al. 2014). PMA has been shown to be nonlethal to *Listeria* (Pan and Breidt 2007), but its lethality to environmental bacteria has not been investigated (Carini et al. 2017). There is some evidence that PMA, while less lethal than its common counterpart, ethidium monoazide (EMA), may still be lethal to some bacteria (Chang et al. 2010). If PMA is, indeed, lethal to some cells, then the results of Carini et al. (2017) would need to be revisited, since PMA treatment would create a false reduction in live cell DNA and an apparent increase in the impact of eDNA. The same could be said if their method inadvertently lysed cells prior to PMA activation.

2.4.2 Evidence that eDNA is not introducing bias

While most studies agree that eDNA is ubiquitous (reviewed in Niemeyer and Gessler 2002; Pietramellara et al. 2009), some studies have determined that its impact on sequence-based community analysis is minimal. For example, Lennon et al. (2017) found that while eDNA comprised an average of 33% of the total bacterial DNA pool in samples across a range of ecosystems, and in one case reached 83%, sequence data revealed that eDNA had no significant impact on estimates of richness, evenness, or either alpha- or beta-diversity. In addition, they developed a set of models to predict the impact of eDNA on apparent community structure, based on the size and composition of both the eDNA pool and the intracellular DNA (iDNA) pool, and the rates of degradation and mortality, respectively, for those pools (Lennon et al. 2017).

They determined that for bias to arise, the distributions of species abundance must be different between the eDNA and iDNA pools (Lennon et al. 2017). Their models showed that when the degradation rate of eDNA is equal across taxa, no bias arises, but that when eDNA derived from distinct taxa degrades at different rates, estimates of community composition will be biased and distinct from the composition of the iDNA pool (Lennon et al. 2017).

There is also evidence that while small amounts of eDNA may be indefinitely stabilized in soils, high-throughput sequencing is not sensitive enough to consistently detect eDNA sequences within 0.9-19.4 days after eDNA enters the soil (See Chapter 1). That work also concluded that the dynamics of eDNA degradation, and perhaps the extent to which eDNA introduces bias to estimates of microbial community structure, vary across gradients of environmental and soil conditions, but that in all cases the sensitivity of the detection method determines in large part how much influence eDNA sequences have (See Chapter 1).

The conclusions of Lennon et al. regarding the minimal impacts of eDNA on community estimates when species abundances are similar between eDNA and iDNA pools are invalidated in situations where absolute abundance of species, rather than relative abundance, are being measured. That is, when using any technique designed to measure absolute abundance, any eDNA present in the sample (regardless of whether it reflects iDNA pool) will introduce bias. Any detectable eDNA in a sample will inflate estimates of absolute abundance, even when estimates of relative abundance are not affected. This leads us to conclude that under many circumstances, eDNA-

driven bias is unlikely, but that in certain situations researchers should be on guard against eDNA-driven bias, and should take steps to minimize its influence.

2.5 *Steps forward*

We have reviewed the currently available literature surrounding the dynamics of eDNA in soils, as well as the potential for eDNA-driven bias in estimates of soil microbial community structure. We focus now on practical steps forward, including techniques to reduce eDNA-driven bias, to harness eDNA in unique ways, and directions that future research should take.

2.5.1 *Reducing eDNA-driven bias*

Under conditions that are likely to result in increased eDNA-driven bias in community estimates, there are approaches that can be taken to remove eDNA or reduce its impact (reviewed in Emerson et al. 2017). The eDNA and iDNA from a sample can be extracted separately (Dell'Anno et al. 2002; Niemeyer and Gessler 2002; Ceccherini et al. 2009; Taberlet et al. 2012; Alawi et al. 2014). A DNase may be used prior to DNA extraction to remove eDNA from a sample (Villarreal et al. 2013; Lennon et al. 2017). DNA-intercalating dyes such as PMA may be used to remove eDNA from downstream analyses (Carini et al. 2017; Emerson et al. 2017). In addition to these laboratory sample-preparation approaches, appropriate experimental design may be key to avoiding eDNA-driven bias in estimates of community structure. To reduce differential eDNA-driven bias among samples, sampling across gradients of moisture, temperature, SOM, tillage regime, habitat, and soil type should all be

minimized. Additionally, sampling across time with very dense resolution has been shown to increase the potential for eDNA-driven bias, depending on the gradient of conditions (See Chapter 1).

2.5.2 Unique opportunities created by eDNA

While sequenceable or stabilized eDNA in the soil may present the risk of biasing estimates of microbial community structure, eDNA also offers two unique opportunities for researchers. The first of those opportunities is the potential to use stabilized eDNA, also called ancient DNA (aDNA), as an archival record of past community structures (Reviewed in Pedersen et al. 2015; Torti et al. 2015; Parducci et al. 2017). For example, aDNA from peri-alpine lake sediment cores was used to estimate changes in cyanobacterial community structure spanning 200 years into the past (Monchamp et al. 2016). That study was able to compare cyanobacterial communities determined from eDNA sequencing with historical records of cyanobacterial communities determined using microscopy. They caution that while there was a strong and significant relationship between the community richness as measured by the two methods, the relative abundances of species were not strongly related (Monchamp et al. 2016). Other studies have found support for the use of sediment eDNA to estimate historical changes in microbial community structure, though all note potential limitations (Corinaldesi et al. 2008; Capo et al. 2015; Capo et al. 2016).

It is important to note that a key limitation when using eDNA as an historical archive is that, due to differences in cell morphology and life history, DNA from some

cells may be protected for a longer period after cell death than others which might affect DNA degradation. Specifically, aDNA from fragile dinoflagellate cells was less abundant at depth (representing 2700 years of sediment deposition) than DNA from more robust diatom cells in a meromictic Antarctic lake (Boere et al. 2011). Significantly, that same study found that fragmentation (and presumably overall degradation) was less for green sulfur bacteria than either of the eukaryotes diatoms or dinoflagellates (Boere et al. 2011).

The second unique opportunity presented by eDNA is the potential to use eDNA as an indicator of the active microbial community in the soil. Several studies support the use of eDNA for community surveys of environmental systems that are easier, cheaper, and more efficient than iDNA-extraction-based techniques (Zinger et al. 2016; Bista et al. 2017). As Zinger et al. (2016) posit, “Active communities continuously release DNA in the environment through biomass turnover, and could hence be better reflected by the extracellular method, although further tests are needed to validate this assumption”. There is a positive correlation between the amount of RNA and DNA that a bacterium produces (Papp et al. 2018), which supports the statement of Zinger et al. (2016). Further research should be done to determine the appropriateness of using eDNA as an indicator of the active microbial community.

2.5.3 *Future directions*

Moving forward, a better understanding of the behavior of eDNA in soils (question 1) may shed light on the extent to which estimates of microbial community

structure are being biased by eDNA (question 2). To address both of these questions, research should attempt to address the following gaps in our current knowledge.

Primarily, much of our understanding of eDNA impacts on community estimates comes from observational studies, and especially given the complexity of soil systems, future studies should focus on controlled experiments to better elucidate the dynamics and impacts of eDNA. Studies tracking eDNA behavior and impacts over longer time periods should be conducted, as most studies only follow eDNA for weeks, not months or years. Studies should also examine the role of eDNA quality (purity, length, source, G + C content, etc.) on degradation/stabilization dynamics. It is unknown whether eDNA molecules from distinct phylogenetic groups experience identical degradation dynamics, though some studies suggest that they do not (Gulden et al. 2005; Pietramellara et al. 2009). Future studies should aim to elucidate the impacts of eDNA source on the extent to which that eDNA introduces bias to estimates of community structure. One of the key stipulations Lennon et al. made when positing that eDNA minimally impacts community structure estimates is that eDNA from diverse sources must degrade at an identical rate (Lennon et al. 2017). Importantly, future research should address the impacts of microbial lifestyle on eDNA dynamics, as one might posit that the eDNA deriving from surface-adhered vs free-living microbes might be subject to distinct fates. We must determine the extent to which microbe ecology impacts the likelihood that its eDNA will be protected or degraded.

Additionally, we lack a solid understanding of the impacts of repeated and/or continual additions of eDNA to soils, as happens in natural systems, and this will be

an especially important arena of future study. Studies should seek to answer questions regarding the behavior of eDNA under repeated additions, the extent to which there exists a fixed quantity of eDNA that can be stabilized in a soil, and whether new inputs of eDNA can displace previously stabilized eDNA in the soil eDNA “reservoir.” In seeking to understand how eDNA impacts estimates of community structure, it may also be important to understand how soil community structure impacts eDNA dynamics. Namely, because some organisms are more or less able to degrade eDNA (Finkel and Kolter 2001; Chimileski et al. 2014; Morrissey et al. 2015), do some microbial communities result in a reduction in eDNA in soils, and a concomitant reduction in eDNA-driven bias, as compared to other communities?

2.5.4 Conclusions

Soil eDNA may introduce bias to estimates of microbial community structure, but evidence surrounding the degree to which eDNA-driven bias is present in sequence-based community estimates is inconclusive. More work must be done to elucidate the details of eDNA dynamics in soils, and to understand the extent to which soil microbial community estimates are influenced by eDNA. This work will not be easy, but is essential to our continued reliance on gene sequence data for understanding microbial communities. We likely do not need to convince you, dear reader, of the vital roles microbes play in soil, and of the potential implications of soil microbial activity on nutrient cycling, carbon sequestration, crop production, pollutant remediation, and innumerable other issues related to humanity’s continued existence on this planet. We do, however, hope to convince you that soil eDNA is a complex

and important topic, and that a comprehensive understanding of eDNA dynamics and impacts will be of benefit to research, current knowledge, and humanity at large. Our ability to effectively capture estimates of microbial community structure is essential to understanding our world, and our ability to understand eDNA in soils is essential to effectively capturing those estimates of microbial community structure.

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